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SCANNING ELECTRON MICROSCOPY OF CHROMOSOMES AND CHROMOSOME FRAGMENTS IN TRANSGENIC RAINBOW TROUT

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Abstract

Chromosomes and chromosome fragments from embryonic offspring of a transgenic rainbow trout were examined using scanning electron microscopy (SEM). SEM is an extremely useful technique for studying the structure of chromosome fragments since little morphological detail is revealed by conventional staining methodologies and light microscopy. The chromosome preparations were processed for SEM by combining an osmium-thiocarbohydrazide-osmium (OTO) technique with 2-4 nm of gold deposition. This technique revealed the organization of individual chromatin fibers in chromosome fragments and intact chromosomes. Both a linear chromosome fragment with a width similar to that of an intact chromatid (approximately 0.60 micrometers) and a spherical chromosome fragment with a diameter slightly greater than the width of an intact chromatid (0.66 micrometers) were observed in metaphase chromosome preparations. A connective fiber (200-300 nm in diameter) between a chromosome fragment and a host chromosome was observed. Interconnecting fibers (approximately 30 nm in diameter) between chromosomes, between chromosomes and fragments, and between sister chromatids were observed in every cell examined. We conclude that SEM permits a detailed analysis of chromosome fragment structure and the nature of chromosome-fragment associations that cannot be obtained using conventional light microscopy techniques.

Key words: scanning electron microscopy, chromosomes, chromosome fragments, rainbow trout

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Introduction

Little information is currently available regarding the ultrastructure of fish metaphase chromosomes. One scanning electron microscopy (SEM) investigation of fish chromosomes revealed few details of the organization of chromatin fibers (Webb, 1974). In contrast, the higher order structure of mammalian metaphase chromosomes has been extensively studied; a model of metaphase chromosome structure involving helical coiling of a 200 nm chromatin fiber (which is composed of a looped 30 nm chromatin fiber) has been proposed by Rattner and Lin (1985) and Taniguchi and Takayama (1986).

The purpose of this study was to examine the chromatin organization of trout chromosomes and compare the chromatin structure of heritable trout chromosome fragments with intact trout chromosomes in offspring of transgenic trout. The transgenic trout were produced by fertilizing rainbow trout eggs with irradiated brook trout sperm, and then heat-shocking the eggs to induce second polar body retention (Thorgaard, 1986, and Disney et al., 1987) in order to determine whether active, foreign genes of interest could be introduced into rainbow trout. Some of the chromosome fragments that we observed in our transgenic trout persisted through adulthood, and were heritable in backcross generations (Disney et al., 1988). Using conventional Giemsa staining and light microscopy, the chromosome fragments often appeared to be spherical in shape and closely associated with an intact chromosome. In order to determine if actual connective fibers existed between chromosome fragments and host chromosomes and whether broken chromosome ends were indeed fusing to form circular fragments, we employed SEM and a fixation technique described by Allen et al. (1986a, b) that permits visualization of chromatin fibers from air-dried chromosomes.

Materials and Methods

Experimental fish

Chromosome preparations from two nine-day embryos which resulted from a cross between a transgenic female rainbow trout (Salmo gairdneri, RT) and a normal male RT were examined. The transgenic parent was derived from a cross described by Thorgaard (1986). Briefly, this cross involved fertilization of RT eggs with brook trout (Salvelinus fontinalis, ST) sperm that had been irradiated with ^{60}Co. The eggs were then heat-shocked for 10 min

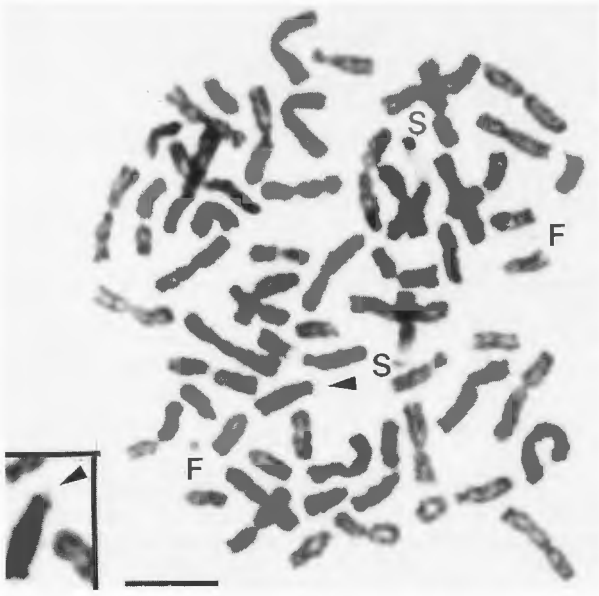

Figure 1. Light photomicrograph of a mitotic metaphase cell from an offspring of a transgenic RT. Chromosome fragments inherited from a transgenic parent are indicated by an 'F'. Satellite chromosomes are indicated by an 'S'. RT normally have 104 chromosome arms; there are 105 chromosome arms in this figure. The arrowhead indicates a chromosome arm that has no homologous partner and is probably of ST origin. There is a small, lightly stained protrusion from the end of this chromosome arm that may correspond to the 200 nm chromatin fiber observed extending from the terminus of a chromosome arm with SEM (Fig. 2F). An enlargement of this chromosome is indicated by an arrowhead in the lower left-hand corner of the photomicrograph. Bar equals 5 micrometers.

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at 29°C to induce retention of the second polar body. The resulting embryos had two chromosome sets from the female parent and a limited amount of genetic material (in the form of autonomous chromosome fragments) from the male parent.

Preparation of embryos

The embryonic offspring of the cross described above were prepared for chromosome analysis using a method described by Thorgaard et al. (1981). Briefly, this involved dissecting the embryos from the chorion while they were in physiological saline, disrupting the yolk sac, and incubating the embryos in media with 25 μg/ml colchicine for five hours. The embryos were then placed in a hypotonic solution (0.56% KCl) for 30 min, fixed in 3 methanol : 1 acetic acid and stored at -20°C.

Chromosome preparation for light microscopy

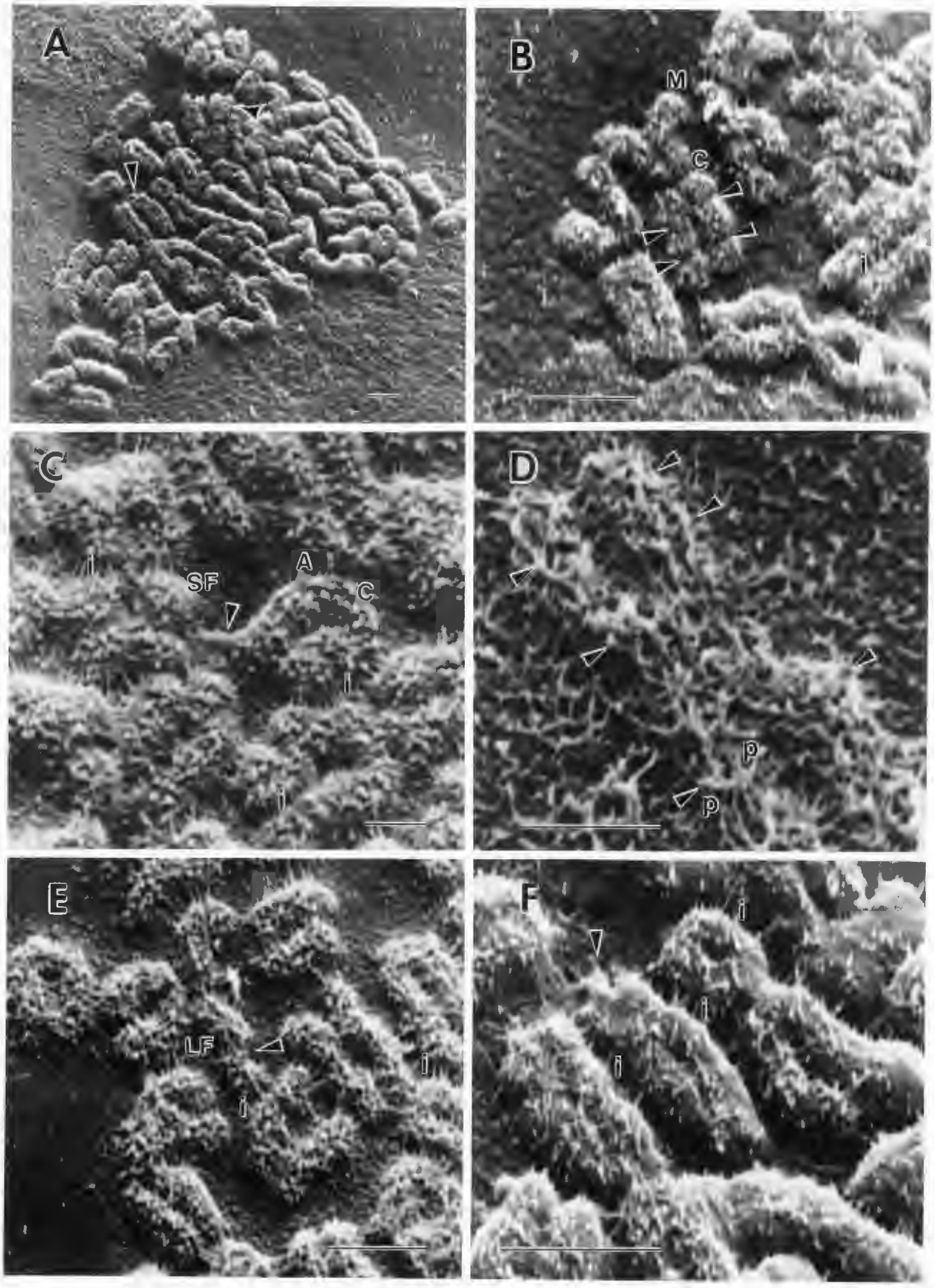
New glass slides and coverslips were soaked in a 1:1 solution of 95% ethanol and concentrated HCl for at least 24 hours, then placed in cold ethanol for at least 15 min and wiped dry with a piece of cheesecloth. This facilitated chromosome spreading and prevented the gold coating from flaking off of SEM preparations.

Chromosome spreads were prepared from embryos as described by Kligerman and Bloom (1977). Embryos were removed from the methanol:acetic acid fix, blotted and placed in 45% acetic acid, and macerated until a cell suspension was obtained. A drop of suspension was placed onto a clean glass slide that had been heated to 45°C on a slide warmer. The drop was withdrawn into a pipette leaving a ring of cells on the slide. Slides were stained in 3% Giemsa in Gurr buffer (pH = 6.8) for 30 min and photomicrographs were made with an Olympus BH-2 light microscope.

Figure 2 (on the facing page). SEM photomicrographs of mitotic metaphase cells from two offspring of a transgenic RT. Bars all indicate 2 micrometers.
A). The arrowhead on the left indicates the same chromosome arm enlarged in Figure 2F. The arrowhead on the right indicates circumferential grooves on the short arm of a metacentric chromosome.
B). Enlarged metacentric chromosome (M) with knobby surface morphology. Arrowheads indicate circumferential grooves. Note the striated appearance of the centromeric constriction indicated by a 'C'. Interconnecting fibers between sister chromatids are indicated by an 'i'.
C). Enlarged acrocentric chromosome (A) with knobby surface morphology. The arrowhead indicates a 200-300 nm connective between the acrocentric chromosome and a spherical fragment (SF). Note the prevalence of interconnecting fibers (designated by an 'i') between chromosomes. The centromere region (C) has no distinctive morphology.
D). Metacentric chromosome that has unravelled to reveal the 30 nm organizational fiber. In some regions two 30 nm fibers run parallel to each other (indicated by a 'p'). Arrowheads indicate areas where the fiber is condensed or compacted.
E). Linear fragment (LF) with an apparent centromeric constriction indicated by an arrowhead. The knobby appearance of the fragment surface is similar to the surface morphology of intact chromosomes. Note again the interconnecting fibers between chromosomes, between sister chromatids and between the fragment and surrounding chromosomes (indicated by an 'i').
F). Enlargement of the chromosome indicated with an arrowhead in Figure 2A. The arrowhead in this figure indicates the 200 nm protrusion extending from a healed terminus of a fragmented chromosome arm. Interconnecting chromatin fibers between chromosomes are again evident and indicated by an 'i'.

Scanning Electron Microscopy

Chromosome preparations were obtained as stated above except that cell suspensions were placed on circular (12 mm) glass coverslips. Coverslips were placed directly onto 38 mm watchglasses and processed as described by Allen et al. (1986a, b), with some modifications. Chromosome preparations were fixed with cold (4°C) 3% glutaraldehyde buffered with 0.1M sodium phosphate (pH = 7.3) for 20 min. Following three rinses in 0.1M sodium phosphate buffer, the samples were postfixed with 1% osmium tetroxide (OsO_4) for 10 min, rinsed three times with distilled water and incubated in a saturated solution of thiocarbohydrazide (TCH) in distilled water for 5 min. Following three rinses in distilled water, the samples received another OsO_4 and another TCH treatment with a final fixation in OsO_4. Chromosome preparations were dehydrated through an ethanol series (30-100%) and critical point dried from CO_2 using a Bomar 1500 critical point dryer. Coverslips were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attached to aluminum SEM mounts using a thick coat of colloidal graphite (Ted Pella Co.) and overlayed with 2-4 nm of gold using a Technics V hummer sputter coater. Samples were examined with an Hitachi S570 SEM at 20 kV using a 60° tilt. Tilt compensation was engaged prior to photography.

Results

Chromosome morphology Whereas only gross morphological differences between chromosomes could be distinguished with the light microscope (Figure 1), the ultrastructure of rainbow trout chromosomes was revealed in this SEM study. We observed that the morphology of rainbow trout (RT) chromosomes was similar to that of mammalian chromosomes. In Figure 2A the cylindrical shape of both metacentric and acrocentric chromosomes is evident. Individual chromatids measure approximately 0.6 micrometers in diameter. Some chromosome arms display circumferential grooves (Figures 2A, 2B) like those characteristic of unbanded mammalian chromosomes (Harrison et al., 1983). The knobby surface of RT chromosomes is apparent in Figure 2B; the knobs averaged about 70 nm in diameter. Similar-sized knobs have been observed in SEM preparations of human metaphase chromosomes and interpreted to be the result of the 30 nm chromatin fiber looping out to the surface and back into the interior (Harrison et al., 1982; Jack et al., 1985; and Allen et al., 1986a).

The centromeres of RT metacentric chromosomes are distinctive in appearance (Figure 2B). Chromatin fibers are stretched between the long and short arms of individual chromatids. Harrison et al. (1983) noted a similar organization of chromatin fibers at the primary constriction of human metacentric chromosomes. There is no distinctive chromatin fiber organization at the centromere of RT acrocentric chromosomes (Figure 2C).

The basic organizational fiber of RT chromosomes is a 30-40 nm fiber as revealed in Figure 2D where the chromatin has unravelled. Some areas of fiber compaction are evident as well as regions where two 30 nm fibers appear to be running parallel through the chromatid.

Chromosome fragment morphology Few morphological details of the chromosome fragments are apparent in light microscope preparations (see Figure 1). SEM preparations of trout chromosomes reveal that some fragments appear linear, while others are spherical in nature. The knobby surface and cylindrical appearance of these fragments indicate that their chromatin organization is similar to that of intact RT chromosomes. The linear fragment in Figure 2E, 1.9 micrometers in length, is shorter than the smallest RT chromosome (2.6 micrometers) and similar in width to the chromatid of an intact chromosome (0.6 micrometers). It has a constriction that is similar in appearance to the centromeres of metacentric RT chromosomes. The fragment has one chromatid; this fragment may not have replicated. Some fragments have a consistently rounded appearance within each cell, similar to double minutes (see Jack et al., 1987). The particular spherical fragment depicted in Figure 2C is 0.66 micrometers in diameter, slightly wider than the chromatid of a metaphase chromosome. It also has no apparent sister chromatid and may not have replicated. Spherical chromosome fragments are often associated with another chromosome. The connective fiber between the fragment and the acrocentric chromosome in Figure 2C is approximately 200 nm in diameter. DuPraw (1970) also described visible connectives between chromosomes and chromosome fragments.

In this particular backcross family, additional chromosome arms as well as smaller chromosome fragments were inherited from the transgenic parent. Some of these arms have distinctive morphologies even at the light microscope level (see arrowhead in Figure 1). A 200-300 nm fiber can be seen extending from the healed end of one such fragmented chromosome (Figures 2A, 2F). Rattner and Lin (1985) frequently observed the end of an internal 200-300 nm fiber (which they interpreted to be the fiber which compacts to form the mammalian metaphase chromatid) extending from the terminus of chromosomes in certain cell lines.

We observed numerous interconnecting fibers (approximately 30 nm in diameter) between RT chromosomes and between fragments and chromosomes in every metaphase cell (see Figures 2B, 2C, 2E, 2F). We also observed interconnecting fibers between mammalian chromosomes in SEM photomicrographs in the review by Harrison et al. (1987), and in articles by Jack et al. (1987), Allen et al. (1986a, b) and Niiro and Seed, (1988), although they are not as prevalent as in preparations of RT chromosomes. Interconnecting fibers are present between sister chromatids of RT chromosomes (see Figure 2B); this is consistent with observations of connective fibers between sister chromatids in human chromosomes (DuPraw, 1970, and Comings and Okada, 1975).

Discussion

The ultrastructure of chromatin organization in RT metaphase chromosomes and chromosome fragments, as revealed by scanning electron microscopy using an osmium tetroxide-thiocarbohydrazide technique, is quite similar to that of mammalian metaphase chromosomes. Rattner and Lin (1985) and Taniguchi and Takayama (1986) described models of mammalian metaphase chromosome structure in which the 30 nm chromatin fiber is looped into a 200 nm fiber which coils and compacts to form a mammalian metaphase chromatid. Rattner and Lin (1985) frequently observed the end of the 200 nm fiber extending from the telomere of chromatids in certain cell lines. We observe a similar sized fiber extending from the healed terminus of a fragmented chromosome arm and a 200-300 nm fiber forming a connective between a chromosome and a chromosome fragment. These connectives, in conjunction with the fact that 70 nm "knobs" appear on the surface of RT chromosomes, suggest that the organization of trout chromosomes is similar to that of mammalian chromosomes.

Although the chromatin organization of trout and mammalian chromosomes is similar, they differ in that trout chromosomes do not G-band (Hartley and Horne, 1985) and display only limited interstitial banding when subjected to restriction enzyme digestion (Lloyd and Thorgaard, 1988). However, trout chromosomes display a longitudinal fluorescent banding pattern in response to replication banding techniques (Delany and Bloom, 1984), as do mammalian chromosomes (for example, see Latt, 1973). The evolution of G-bands is hypothesized to have occurred

much more recently than the temporal arrangement of replicon clusters (Holmquist et al., 1982). The only example of clear euchromatin banding in fish chromosomes is for the European eel (Wiberg, 1983). Medrano et al. (1988) suggest that this G-banding may be related to the observation that DNA from European eels produces bands in CsCl density gradients that are characterized by a high compositional heterogeneity and a strong asymmetry as compared with the species of fish that do not G-band.

Similar correlative DNA data are not available for trout; nonetheless, the pattern of circumferential grooves observed on RT chromosomes in the SEM suggests that some G-banding of trout chromosomes could be possible, especially since grooves enhanced by trypsin treatment in human chromosomes correspond to G-bands in the light microscope (Harrison et al., 1981, 1985). SEM studies involving trypsin treated trout chromosomes may reveal whether trout chromosomes do indeed have the potential to G-band.

The chromatin structure of chromosome fragments in progeny of a transgenic individual is similar to that of intact chromosomes. Although some fragments appear to have a centromere-like constriction, others do not. Associations of fragments with host chromosomes are repeatedly observed and may be a mechanism by which fragments segregate into daughter nuclei. This is the mechanism by which it is hypothesized that acentric double minute chromosomes segregate (Jack et al., 1987). It is interesting that some fragments do not appear to have sister chromatids. Many adult transgenic RT were mosaic for pigment gene expression and foreign isozyme expression (Disney et al., 1987, 1988); failure of fragments to replicate may be one explanation for the observed mosaicism.

Interconnecting fibers between RT chromosomes and sister chromatids may arise during preparation or may reflect a real cytological phenomenon. DuPraw (1970) presented intriguing evidence that non-homologous chromosomes often have physical connections and speculated that chromosome-to-chromosome connectives might play a role in the evolution of chromosome number and morphology. The prevalence of these fibers between RT chromosomes (in comparison to the dearth of fibers between human chromosomes in similarly spread preparations) is particularly intriguing in light of the interesting evolutionary history of salmonids (see Wright et al., 1983). Robertsonian fusion is the primary mechanism of chromosome evolution in these tetraploid derived animals (see review by Hartley and Horne, 1987). Rainbow trout chromosomes undergo extensive multivalent pairing at meiosis (Ohno et al., 1969) which, in males, may result in co-segregation of unlinked genes. Associations of mitotic RT chromosomes are also often observed (see Bolla, 1987).

The structure of segregating chromosome fragments was revealed by comparing their surface morphology to that of intact chromosomes. The SEM analysis of trout chromosomes also revealed some interesting details of trout chromatin organization. A thorough SEM study of trout chromatin structure, involving treating the chromosomes with chemicals such 33258 Hoechst or 5-azacytidine to decondense the chromosomes, might elucidate why differential G-banding has not been obtained with trout chromosomes. Mechanisms involved in chromosome fusion and multivalent pairing might become evident as well.

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Discussion with Reviewers

T.D. Allen: Was an intermediate solvent used between the absolute alcohol and liquid CO_2.
Authors: No.

T.M. Seed: Do the authors have any ideas of the stability of chromosome fragments?
Authors: In light microscopy studies, we often observed fragments in close association with intact host chromosomes (Disney et al., 1988). These observations led us to hypothesize that some fragments may be segregating by association with intact chromosomes. We undertook this SEM study in an attempt to visualize an actual physical connection between fragments and intact chromosomes. In 4 cells, we observed a 200-300 nm connective fiber extending between an apparently acentric spherical chromosome fragment and a host chromosome (for example, see Figure 2C). We have previously observed that chromosomes with NORs (nucleolar organizer regions) are often associated in trout metaphase preparations (unpublished data). This led us to question whether some stable fragments carry NORs (Disney et al., 1988). Indeed, 9 of 14 embryos (from 4 transgenic parents) that were examined for NOR expression had additional active NOR regions either on chromosome fragments or in association with or integrated into host chromosomes. Segregation by association may not be the only mechanism by which chromosome fragments are maintained in these transgenic fish. Certainly, further studies on the structural components of these fragments (i.e. do some fragments have kinetochores and telomeres?) are necessary to fully understand the mechanism of fragment stability.

R.B. Phillips: Did the authors observe any pattern to the interchromosomal fibers? Were the fibers just as numerous in spreads with widely separated chromosomes as in more compact spreads such as the one illustrated?
Authors: We observed no particular pattern regarding the interchromosomal fibers except that they were parallel to each other (i.e. these fibers did not criss-cross). Otherwise, they appeared to be randomly distributed among the chromosomes and chromosome fragments. In more widely separated chromosome spreads, these interchromosomal fibers were not observed, however, fibrous connections between sister chromatids were still evident.

M.E. Delaney: Were the chromosome fragments observed in all cells examined?
Authors: Fragments were not observed in all cells examined, although we are not certain that these individuals were mosaic. Small fragments are easily obscured by intact chromosomes or lost during metaphase chromosome preparation. To address this question we examined anaphase preparations and interpreted anaphase aberrations as being indicative of fragment instability (Disney et al. 1988). Siblings of the embryos examined in this SEM study did indeed display significant numbers of anaphase aberrations (lagging chromosomes and bridge formations) as compared to control embryos. The association of fragments with intact chromosomes might interfere with the ability of chromosomes to segregate properly.

M.E. Delaney: Was a centromere-like constriction ever observed within the spherical fragments?
Authors: The spherical fragment was observed using SEM in 12 cells and a centromere-like constriction was never apparent.

M.E. Delaney: Does the lack of distinctive centromeric organization observed in the acrocentric chromosomes provide evidence that these chromosomes should be designated telocentric? Was this observed in all acrocentrics? How does this result compare with the centromere structure observed by SEM of mammalian acro- and telocentrics?
Authors: We have made light microscope and SEM preparations of human chromosomes from peripheral lymphocyte cultures. Human acrocentric chromosomes did not always appear to have a primary constriction in our light microscope preparations but all 10 human acrocentric chromosomes always had a primary constriction in our SEM preparations. In our trout chromosome preparations, however, we observed only 2-4 acrocentric chromosomes with primary constrictions (we typically refer to these chromosomes as subtelocentrics) and the remaining acrocentric chromosomes truly had no distinctive chromatin fiber organization at the centromere. Therefore, "telocentric" might be a better designation for these chromosomes.

J.B. Rattner: Are the smallest spherical fragments comparable in size to the reported size of chromomeres? I wonder if there is a minimal size for a chromosome fragment.

Authors: We estimate a trout chromomere to be approximately 0.3-0.5 micrometers in length. The smallest spherical fragment that we measured was slightly larger (0.66 micrometers) than the estimated size of a chromomere. We are also interested in what constitutes a minimal autonomous chromosome fragment. We suggest that this in vivo chromosome-mediated gene transfer system might prove useful for defining the minimal requirements of a functional higher eukaryotic chromosome.

T.D. Allen: Is it possible to produce spreads in which individual chromosomes are more spaced? If so, firstly there will be more ultrastructural information available, as some is masked by adjacent chromosomes. Secondly, in mentioning interconnecting fibers, we feel that these fibers are probably slightly dispersed chromatin loops at the chromosome periphery, and tend to be merely 'tangled' in those of adjacent chromosomes. Thus if adjacent chromosomes were further away, the nature of this dispersed surface chromatin might become apparent.
Authors: Yes, it is possible to produce spreads in which individual chromosomes are more spaced. In such preparations, the interconnecting fibers could not be seen and may have broken during the air-drying and spreading process. Although it is possible that these fibers are dispersed chromatin loops at the chromosome periphery that have become 'tangled' with dispersed chromatin loops of adjacent chromosomes, we never observed "intermediately" spread chromosome preparations with overlapping points of entanglement. The interconnecting fibers were either present in parallel between chromosomes or were not present.