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Toward a physical map of *Aedes aegypti*

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Abstract

Labelled recombinant cosmids were used as *in situ* hybridization probes to *Aedes aegypti* metaphase chromosomes. The cosmid probes yielded paired signals, one on each arm of sister chromatids, and they were ordered along the three chromosomes. In total, thirty-seven different probes were mapped to the three chromosomes of *Ae. aegypti* (2n = 6): twenty-eight to chromosome 1, six to chromosome 2, and six to chromosome 3. These results represent an initial stage in the generation of a physical map of the *Ae. aegypti* genome.

Keywords: *Aedes, aegypti*, FISH, map, physical.

Introduction

The yellow fever mosquito, *Aedes aegypti*, is a vector of debilitating and lethal pathogens affecting humans and other vertebrates (Anon., 1991), and it is one of the better genetically characterized mosquito species. Seventy-seven isozyme and morphological mutant marker loci have been placed into three genetic linkage groups (Munstermann & Craig, 1979; Munstermann, 1990). In addition, an RFLP linkage map containing fifty markers, which identify fifty-three loci across three linkage groups spanning 134 map units, has also been reported (Severson *et al.*, 1993). The haploid genome has been estimated to be approximately 0.83 pg (~780 Mb) (Rao & Rai, 1987; Cockburn & Mitchell, 1989; Warren & Crampton, 1991), which is distributed as two metacentric (I and III) and one submetacentric (II) somatically paired metaphase chromosomes (2n = 6) (Rai, 1963).

Aedes aegypti differs from anophelines and drosophilids because the *Ae. aegypti* genome exhibits short period dispersion of repetitive elements (Cockburn & Mitchell, 1989). Although polytene chromosomes have been prepared from *Ae. aegypti* tissues and a polytene cytogenetic map has been proposed, slides of polytene chromosomes were extremely difficult to produce consistently where only one slide in 200 was usable (Sharma *et al.*, 1978). Thus, *Ae. aegypti* polytene chromosomes are not usable as a consistent source for physical mapping. The lack of usable polytene chromosomes also means that chromosomal deletions, inversions and translocations cannot be detected, located, or followed among *Ae. aegypti* populations. In contrast, the *An. gambiae* polytene chromosomes have been amenable to a 'bottom-up' approach to physical mapping, such as microdissection as pioneered in *Drosophila* (Wesley *et al.*, 1990; Kafatos *et al.*, 1991), and a low-resolution physical map for *An. gambiae* has been reported (Zheng *et al.*, 1991).

The challenge in genomic physical mapping is to map a large number of clones at intervals spanning the region(s) of interest and to accomplish the task rapidly and efficiently (Ward *et al.*, 1991). Recent advances in fluorescence *in situ* hybridization (FISH) and digital imaging microscopy have met this challenge, and the approach has had a profound impact on high resolution physical mapping and gene identification in humans (Lichter & Ward, 1990; Lawrence, 1990; Trask, 1991; McNeil *et al.*, 1991; Hozier & Davis, 1992). Although the ability to resolve two signals (probes) in terms of base pairs, depends upon the material examined (McNeil *et al.*, 1991; Ward *et al.*, 1991; Hozier & Davis, 1992), probes are mapped initially to metaphase chromosomes at a resolution of approximately 1 Mb. Distances between probe combinations may be measured and ordered between 50 kb and 4 Mb when interphase nuclei are the hybridization target (van den Engh *et al.*, 1992; Yokoka *et al.*, 1995). Extended chromatin mapping (or direct *in situ* hybridization, DIRVISH) to relaxed, extended chromatin fibres, increases the resolution between signals to approximately 1 kb (Parra & Windle, 1993; Haaf & Ward, 1994).

Herein we apply FISH in physical mapping of the *Ae. aegypti* genome. The *Ae. aegypti* metaphase chromosomes were prepared from the ATC-10 cell line and these are characterized morphologically. A 'top-down' physical mapping strategy for the *Ae. aegypti* genome is discussed.

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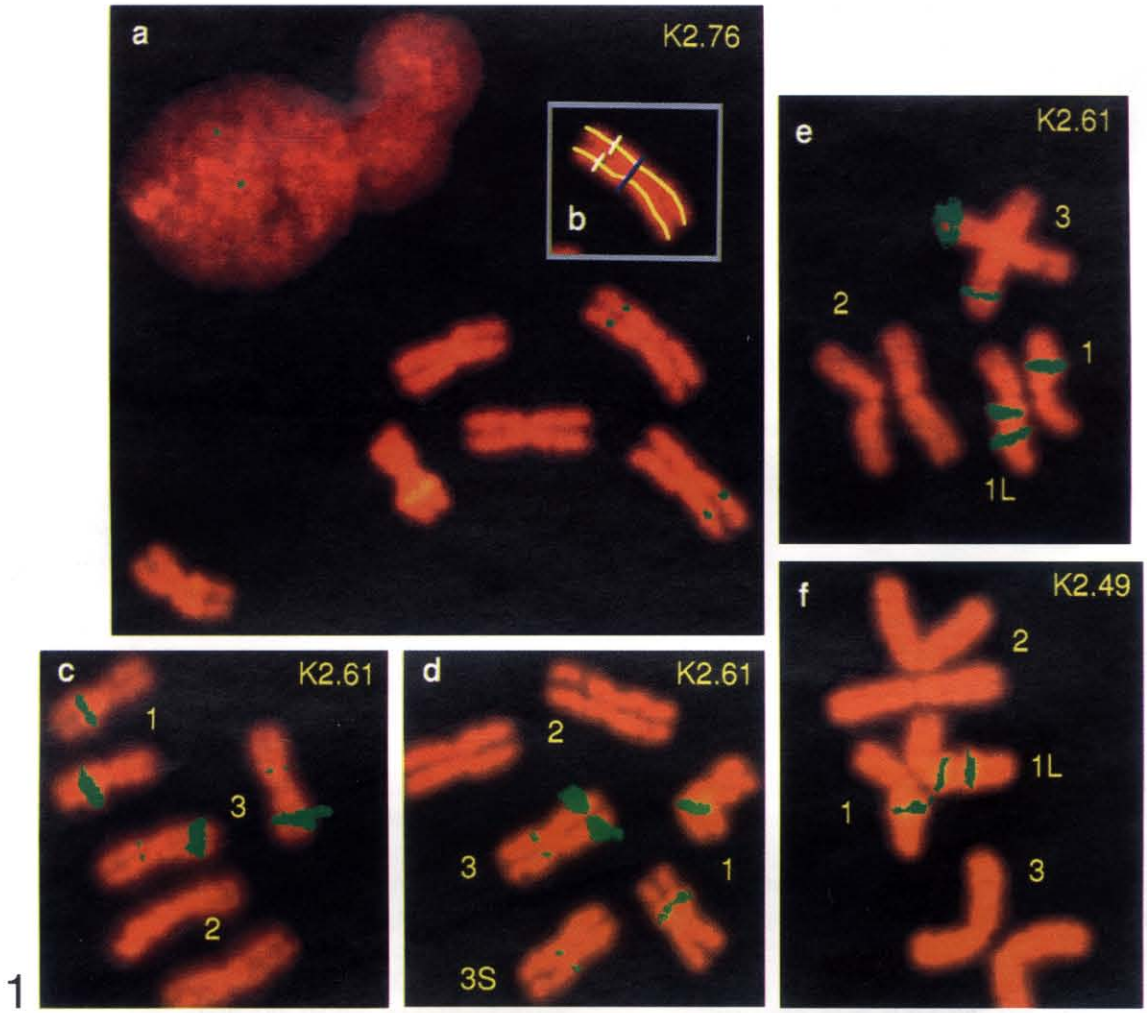
a
K2.76
b
e
K2.61
3
2
1
1L
f
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1L
1
3
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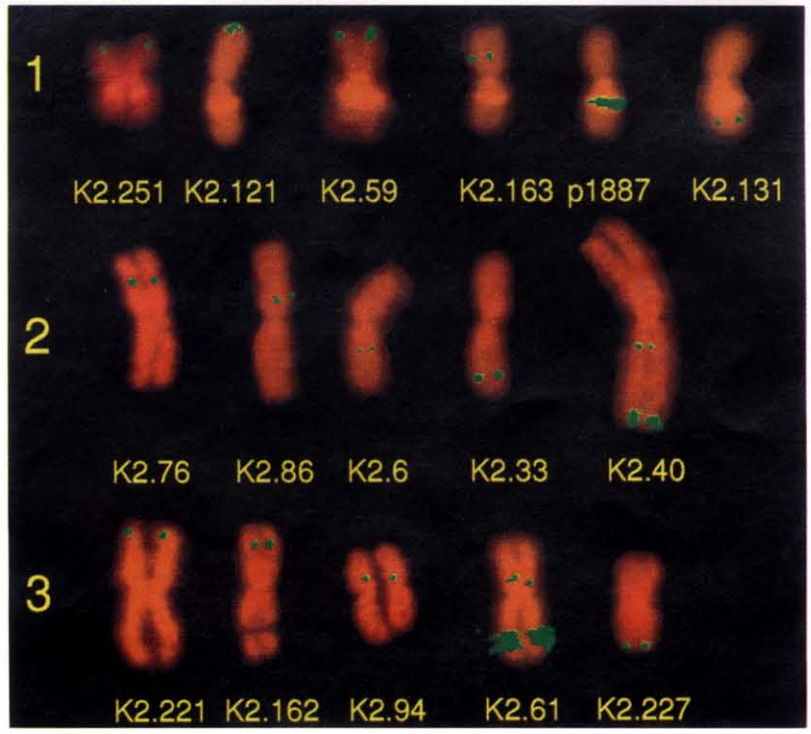
2
3
1
3S

1


1
K2.251 K2.121 K2.59 K2.163 p1887 K2.131
2
K2.76 K2.86 K2.6 K2.33 K2.40
3
K2.221 K2.162 K2.94 K2.61 K2.227

2

Table 1. Statistics for ATC-10 metaphase chromosomes.

Chromosome	*N* (153)	Average μm pter-centromere	SD	Average μm centromere-qter	SD	Average μm total	SD	Average % pter-centromere	Average centromere-qter
1	68	2.95	0.37	3.42	0.43	6.37	0.73	46.33	53.67
1L	5	3.41	0.57	5.10	0.77	8.51	1.31	40.05	59.95
2	52	4.11	0.79	4.51	0.91	8.62	1.67	47.70	52.30
3	8	4.07	O.40	4.26	1.01	8.33	1.38	49.38	50.62
3S	20	3.36	0.57	3.67	0.59	7.03	1.15	47.82	52.18

1L and 3S represent anomalous metaphase chromosome morphologies; see text for discussion.

Results and Discussion

FISH mapping to metaphase chromosomes requires high-quality chromosome preparations with high mitotic indices. It also requires tagged probes which contain sufficient single copy sequence such that a fluorescent signal can be detected. We have found that chromosome preparations made from the *Ae. aegypti* cell line, ATC-10, are well suited to FISH analysis because the chromosome morphology in preparations was consistent and reproducible. Hybridization of cosmid clones gave paired signals on the sister chromatids of greater than 90% of all spreads examined. As an example, the signals observed on chromosome 2 when the cosmid probe K2.76 was hybridized to propidium iodide stained ATC-10 metaphase chromosomes is presented (Fig. 1a). The signal position was then measured as a fractional length from the p terminus (FLpter) along the chromosome (Fig. 1b). Using the FISH approach, one plasmid and thirty-six cosmid probes were labelled, hybridized, and assigned to metaphase chromosomes. A composite image of these FISH results was prepared, clearly revealing the chromosome assignment and the relative map order of the probes (Fig. 2). FLpter measurements were made of the signal position on the FISH probed metaphase chromosomes and are graphically depicted as an *Aedes aegypti* physical map (Fig. 3). Along with the FLpter measurements, ATC-10 metaphase chromosome lengths and centromere positions were also measured (Table 1). Interphase nuclei, which are present in all metaphase chromosome preparations, exhibited either two or four signals depending upon their phase in the cell cycle (Fig. 1a), and therefore they can be used to obtain higher resolution ordering of clones when multiple probes or probes in combination are used (Ried *et al.*, 1992).

The pter was assigned to the smallest arm of the meta to submetacentric chromosomes (Table 1). Although the central position of the centromere in the three chromosomes made distinguishing the pter difficult, propidium iodide staining of metaphase chromosomes resulted in a consistent, light band on the q arm of chromosome 1. One q arm of chromosome 3 was missing a fragment in most preparations, and thus it represented a karyotype anomaly (3S in Table 1). FISH results with cosmid probe K2.61 are illustrative of this observation. For example, K2.61 hybridized to chromosome 1 detecting a large block on the q arm, which co-localized with the propidium iodide band. K2.61 also hybridized to another large block on the q arm of chromosome 3 with a minor signal in the p arm (Fig. 1c). In the majority of the chromosome preparations, K2.61 yielded a pattern of fluorescence in the q arm of chromosome 3 where the signal was present on one chromosome and missing on the other. Given that signal was present on both chromosomes, albeit with a lower frequency, and that breaks in chromosome 3 were seen (Fig. 2, second chromosome from the left), it is likely that the terminal portion of the q arm contains a fragile site which is prone to break during metaphase chromosome preparation. Nevertheless, this observation allowed consistent orientation of chromosome 3 (Fig. 1d). Chromosome 2 was the largest of the three chromosomes and it was routinely easy to identify

Figure 1. Photomicrographs of fluorescence *in situ* hybridization with Cot1 suppression of labelled cosmid clones to *Aedes aegypti* metaphase chromosome preparations are represented. The slides were counterstained with propidium iodide and the biotin-labelled cosmid signals were detected with an avidin–FITC conjugate. Digital images were collected, enhanced, colourized and merged. A typical FISH mapping result is represented using the cosmid K2.76 probe where paired signals may be seen on the sister chromatids of chromosome 2 and signals are also seen in a G1 interphase nucleus which is located in the upper left (Fig. 1a), and an example of the measurements made is also presented in the inset (Fig. 1b). While the ATC-10 chromosome preparations were an excellent source for FISH mapping, several chromosomal anomalies were found and these anomalies are well-illustrated using cosmids clones K2.61 and K2.49 as probes (Figs 1c–1f). The 3S anomaly (Fig. 1d) was seen frequently and the 1L anomaly (Figs 1e and 1f) was seen infrequently (Table 1).

Figure 2. A composite of selected chromosomes taken from digital images of *Aedes aegypti* metaphase chromosomes and FISH. Images have been selected and aligned to reveal probe map order on the chromosome (see Fig. 3). Samples were stained with propidium iodide and hybridized with the biotin-labelled probe noted. The propidium iodide band in the q arm is seen in all samples except plasmid p1887, where specific hybridization co-localizes with the propidium iodide band. Note the paired hybridization signals on sister chromatids starting at the top (pter) going to the bottom or qter (left to right, see also Fig. 3).

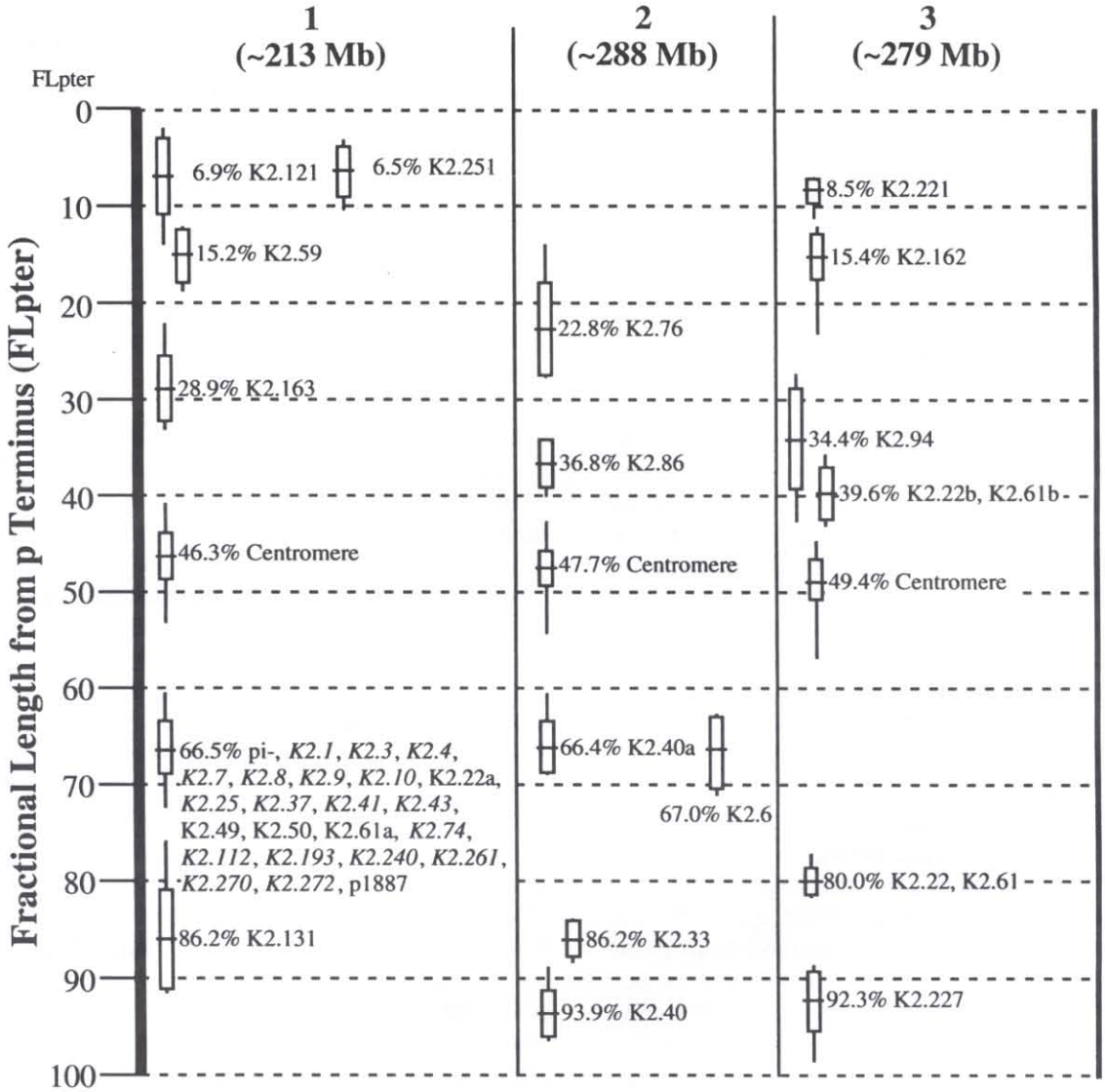


Figure 3. A graphic depiction of the *Aedes aegypti* physical map where position of the mapped clones are indicated as a fractional length from the p terminus (smallest chromosome arm) or FLpter. For mapped probes, the horizontal bar marks the map position with the box representing the standard deviation of the measurement and the vertical bar representing the high and low values recorded. The italicized clones at 66.5% FLpter were placed by inspection. Chromosome size in Mb are estimates using 0.83 pg (Warren & Crampton, 1991) or 780 Mb and the relative length of the three chromosomes.

the pter. In addition to the 3S anomaly (short arm of chromosome 3) (Table 1), a long q arm was seen infrequently for chromosome 1 (1L in Table 1, Figs 1e and 1f). Since K2.61 hybridized strongly with the q arm of chromosome 3 and cross-hybridized with the propidium iodide band (FLpter 66.5%) on chromosome 1 (Figs 1 and 2), it will be used to mark and orient the chromosomes in future FISH mapping.

The p1887 plasmid probe, which is approximately 1.7 kb, also co-localized with the propidium iodide band at 66.5% FLpter. End-sequence analysis indicates that p1887 contains the 18S ribosomal gene (S. Brown, unpublished). Therefore the propidium iodide band probably represents the repetitive ribosomal cistron region which has been seen in autoradiographic *in situ* hybridization in *Ae. aegypti* (Kumar & Rai, 1990) and by FISH in other culicine mosquitoes (Marchi & Pili, 1994). Whether this region corresponds to the *NO* locus, which has been placed on the genetic linkage map, remains to be demonstrated (Munstermann & Craig, 1979; Munstermann, 1990). An unusually large number of the cosmid probes mapped to 66.5% FLpter, suggesting that the one amplification of the cosmid library (K2) resulted in a library skewed for those clones. We have no explanation for this observation other than these clones grew well upon amplification.

Since the protocols described here for metaphase chromosome preparations from the *Ae. aegypti* (ATC-10) cell line consistently yielded high quality chromosomes suitable for FISH, the cell line will continue to be used as the source for chromosome material for *Ae. aegypti* physical

genomic mapping. Using the cell line as a map standard, rather than the whole insect, has a number of benefits: continuous availability of materials, consistency in preparations and genetic background, high mitotic indices, fine scale mapping via extended chromatin mapping (or DIR-VISH) is possible (Parra & Windle, 1993; Haaf & Ward, 1994), and the cell line is available from the American Type Culture Collection.

As the mapping standard, ATC-10 will provide the needed reference point to which map data developed on whole mosquitoes may be compared. In addition to establishing an initial reference FISH physical map for the *Ae. aegypti* genome, this approach will produce and identify reagents which will allow chromosomal deletions, inversions and translocations to be detected and located in field populations of *Ae. aegypti* and other mosquitoes, facilitate gene identification through map-based or positional cloning strategies, and provide probes for developmental studies in the whole insect.

Experimental procedures

Cell line

The cell line used as a source of genomic DNA and metaphase chromosome preparations was the *Aedes aegypti* continuous cell line ATC-10 (Singh, 1967), which is available from the American Type Culture Collection designated as ATCC CCL-125. The cell line was maintained in 150 cm^2 flasks and grown in Leibovitz's L-15 media (GIBCO-BRL Life Technologies, Inc.) with 100 units/ml of penicillin and 100 μg/ml streptomycin (GIBCO-BRL Life Technologies, Inc.) and 10% fetal bovine serum. The cell cultures were split 1:2 once a week.

DNA

Ae. aegypti genomic DNA was isolated from the ATC-10 cell line following procedures described elsewhere (Ausubel *et al.*, 1987).

Cosmid libraries

A cosmid library was constructed using ATC-10 genomic DNA, the SuperCos-1 cosmid vector kit, and the Gigapack II XL packaging extract (Stratagene Cloning Systems). The SuperCos-1 vector, a 7.6 kb cosmid, contains two *cos* sites, a cloning region flanked by T3 and T7 bacteriophage promoters, *Not*I sites flanking these promoters, a *neo* marker, and an *amp* resistance gene. The packaging system preferentially selects for recombinants 47–51 kb in total size. The library was constructed following protocols of the supplier (Stratagene Cloning Systems). Briefly, the genomic DNA was partially digested with *Mbo*I to yield segments with an average size of approximately 50 kb, as determined by fragment mobility in a 0.7% agarose gel (Stratagene Cloning Systems protocol, SuperCos 1 Cosmid Vector Instruction Manual, 14 February 1991). The genomic DNA was ligated into the *Bam*HI site of the cosmid vector and packaged. The library was titered, amplified once, and stored (Stratagene Cloning Systems protocol, Gigapack II Packaging Extract Instruction Manual, 10 May 1991).

The transduced bacteria were plated on LB-*amp* plates to yield well-isolated recombinant colonies. The colonies were picked and grown in tubes containing 3 ml LB-*amp* for 6 h and an aliquot of the bacterial culture was frozen at −70°C in 18% glycerol. The cosmid DNA was isolated from the remainder using a modified boil-prep procedure (Holmes & Quigley, 1981) as suggested by the supplier (Stratagene Cloning Systems, SuperCos1 Cosmid Vector Instruction Manual, 14 February 1991). The procedure was doubled so that a 3 ml culture was processed in each tube. After the boiling step and before the precipitation step, two phenol–chloroform extractions were performed. The precipitated DNA was dissolved in 25 μl of water. Since *Not*I restriction of the recombinant cosmid excises the insert, 3 μl of the DNA was digested and separated by electrophoresis in a 1% agarose gel using a CHEF-DR II electrophoresis system (Bio-Rad Laboratories, Inc.) to size the inserted *Ae. aegypti* fragments. The CHEF-DR II run parameters were: Initial A time = 1; Final A time = 6; Ratio = 1; Run time 15 h; 200 V and the electrophoresis buffer was 0.5 × TBE pH 8.3 (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA pH 8.0). The molecular weight markers ranged from 1503 to 48,502 bp (United States Biochemicals, Inc., Cat. 73320).

Metaphase chromosomes

Metaphase chromosomes were prepared from *Ae. aegypti* ATC-10 cell cultures using standard cytogenetic procedures. ATC-10 cells were split 1:2 and grown in 75 cm^2 flasks for 48 h prior to treatment. The cultures were starved in media without fetal bovine serum for 24 h, and they were incubated in media with excess fetal bovine serum (35%) and colcemid (0.1 μg/ml, GIBCO-BRL Life Technologies, Inc.) for an additional 24 h. The cells were harvested, passed through a 20 gauge needle and syringe, and exposed to 7 ml of a hypotonic pre-warmed 75 mM KCl solution for 5 min at 37°C. 3 ml of freshly made fixative (methanol:acetic 3:1) was added to the suspension and the sample was pelleted for 5 min in a benchtop centrifuge. The supernatant was removed, 4 ml of fixative was slowly added to the cell pellet, and the cells were fixed for 30 min on ice. The cells were washed four times with 4 ml of fixative. The final cell pellet was resuspended in 200 μl of fixative and the cells were dropped on cold glass slides. The slides were dehydrated through an ethanol series (70, 90 and 100%), air dried, and stored *in vacuo*.

Cot1 fraction

Aedes aegypti DNA was isolated from the ATC-10 cell line using the GNOME DNA isolation kit (BIO 101 Inc.). The DNA was diluted in TE buffer (10 mM Tris pH 7.5, 1 mM EDTA pH 8) to a final concentration of 100 μg/ml. The procedure for isolating a reanealled fraction, enriched for repetitive elements, where C_0t (mole × s/liter) equals 1, was followed (Britten & Kohne, 1968). Briefly, 1 mg of DNA was sheared by ten cycles through a 27-gauge needle using a 5 ml syringe. The DNA sample was heated at 95°C for 10 min and allowed to reanneal for 1 h at 37°C. S1 nuclease (500 units/100 μg DNA) was added, and the sample was incubated at 37°C for 30 min. EDTA was added to stop the reaction and the sample was extracted with phenol chloroform. The sample was precipitated and resuspended in 500 μl TE buffer pH 7.5, and

a 5 μl aliquot was examined by 1% agarose gel electrophoresis to determine the size range of the fraction. The target size range was between 100 and 500 bp. If the fraction was too large, it was treated with DNase following standard protocols until the sample was within the appropriate range.

In situ *hybridization*

Laboratory protocols for *in situ* hybridizations have been described in detail elsewhere (Nisson *et al.*, 1991; Ausubel *et al.*, 1987). Briefly, recombinant cosmid DNA was labelled with biotin-11-dUTP using standard nick translation procedures. The cosmid DNA was prepared using the boil-prep method and 20 μl of DNA was used in each labelling (approximately 0.5–1 μg). Since the recombinant cosmids also contained repetitive *Ae. aegypti* DNA elements, a Cot1–suppression–hybridization step was required. This step removed the repetitive elements by annealing with the Cot1 fraction, which was in excess; and therefore the number of labelled repeats which could hybridize to the chromosomes was reduced. The labelled probe, excess *Ae. aegypti* competitor DNA (Cot1 fraction, 2 μg) and salmon sperm DNA, which was also sized to 100–500 bp (10 μg), are precipitated, denatured at 80°C for 10 min, and allowed to reanneal at 37°C for 30 min. Slides containing metaphase chromosomes were treated separately to denature the chromosomal DNA, the pre-annealed probe was added to the slide, and hybridization occurred overnight. The excess probe was removed from the slides by several washes. The slides were blocked with 3% bovine serum albumin in 4 × SSC and the biotinylated probe was detected with FITC-conjugated avidin; any excess was removed by a brief wash. The slides were counterstained with propidium iodide (0.2 μg/ml) and stored at 4°C until examined optically.

Microscopy and digital imaging

Propidium iodide and FITC stained digital images were captured with a 63 × Zeiss objective using a cooled array CCD collector (CH-250, TK512, Photometrics Inc.) mounted on a Zeiss Axioskop microscope (Carl Zeiss Inc.) equipped with compatible band-pass filters. The image capture was controlled by the software package CCD Image Capture running on a Macintosh computer. The captured digital images were enhanced using NIH Image and then colorized and merged using the Gene-Join MaxPix software on a Macintosh computer (Quadra 950 or PowerPC 8100/80). Chromosomal lengths and features were measured electronically on the final merged image using the Segmented Ruler software. The public domain NIH Image program (written by Wayne Rasband at the US National Institutes of Health) is available from the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, part number PB93-504868). The Gene Join software package which includes CCD Image Capture, Gene-Join MaxPix, and Segmented Ruler was developed in the Ward laboratory and is available through Yale University (Office of Cooperative Research, 246 Church Street, Suite 401, New Haven, CT 06510, USA).

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