

Characterization of whole genome radiation hybrid mapping resources for non-mammalian vertebrates

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ABSTRACT

Radiation hybrid panels are already available for genome mapping in human and mouse. In this study we have used two model organisms (chicken and zebrafish) to show that hybrid panels that contain a full complement of the donor genome can be generated by fusion to hamster cells. The quality of the resulting hybrids has been assessed using PCR and FISH. We confirmed the utility of our panels by establishing the percentage of donor DNA present in the hybrids. Our hybrid resources will allow inexpensive gene mapping and we expect that this technology can be transferred to many other species. Such successes are providing the basis for a new era of mapping tools, in the form of whole genome radiation hybrid panels, and are opening new possibilities for systematic genome analysis in the animal genetics community.

INTRODUCTION

Both the chicken (*Gallus domesticus*) and the zebrafish (*Danio rerio*) have emerged as important model organisms for studying the genetics of growth, reproduction and development. However, an insufficiently developed mapping infrastructure for many non-mammalian species limits further application of molecular genetic techniques. Attention has been focused towards providing genome-wide mapping resources to utilize genetics to investigate biology. These investments are a pre-requisite for further genetic and functional characterization of the dazzling number of both zebrafish (1) and chicken (2) mutant phenotypes. In addition, since both poultry and fish are of major agricultural importance, the application of rapid mapping approaches will be invaluable for the implementation of marker-assisted selection programs (3). While genetic markers are now more widely available for many organisms, positioning these markers in both fish and birds has relied with limited success on recombination mapping, often based on single crosses (4–6). Recently, radiation hybrid panels have been recognized as landmarks in the human genome

community, since almost any marker (even when non-poly-morphic, including cloned genes) can easily be placed at its exact chromosomal location (7). Radiation hybrid panels can be tailored to different mapping resolutions and 'whole genome' radiation hybrids (WGRH) can position markers on all chromosomes at once. This not only allows for rapid and inexpensive construction of comprehensive genome and gene maps, but, more importantly, integration of physical with genetic maps is made possible. Such resources help accelerating cloning strategies, as well as extending comparative gene mapping through exchange of mapping data between genomes of different species. To date, a collection of WGRH containing the donor genome as small DNA fragments can only be generated between mammalian species (8–11). It was not known whether WGRH could also be created for non-mammalian classes such as fish and birds. In this paper, we demonstrate that comprehensive chicken and zebrafish WGRH panels can be obtained by fusion to hamster cells, the same method that was used for generating human panels (8). We have investigated several important properties of the chicken and zebrafish panels and conclude this report by showing the utility of our panels for mapping purposes.

MATERIALS AND METHODS

Cell lines

The donor cell line AB9 was derived from fin amputation of adult zebrafish AB strain. The fins were extensively washed in polyantibiotic solution (12) and cells were maintained in DMEM with 15–20% foetal calf serum, penicillin/streptomycin and glutamine at 28°C in 5% CO₂ (13). The AB9 cell strain has been submitted to the American Type Culture Collection (Rockville, MD). The chicken donor cell lines F23 and F21 are early passage primary fibroblastic cell lines derived from feather pulp of Ross 1 chickens and were maintained in Hams F10 (Sigma) supplemented with 15% foetal calf serum, 25 mM HEPES buffer, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B at 40°C in 5% CO₂. The recipient cell line Wg3H (14) is a hypoxanthine-guanine phosphoribosyltransferase deficient

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(HPRT⁻) hamster line; this cell line generates wild-type revertants at very low frequency. The recipient cell line A23 (15) is a thymidine kinase deficient hamster fibroblast cell line.

Generation of radiation hybrids

The chicken–hamster whole genome radiation hybrids were generated as described elsewhere (11), as were the zebrafish hybrids, except that 10⁸ AB9 cells were fused to the same number of Wg3H cells. No colonies were observed in the zebrafish controls (irradiated AB, Wg3H grown in HAT), although a low frequency was observed in the chicken fusions with Wg3H. Approximately 3 weeks after the fusion, colonies were picked into 6-well plates and grown in T75 flasks for DNA extraction. All fused cells were maintained at 37°C.

FISH

Metaphase spreads from chicken–hamster and zebrafish–hamster hybrids and chicken cells were prepared by standard cytogenetic techniques. FISH was performed as described (16). Essentially, 200 ng total genomic DNA labelled with biotinylated 16-dUTP (Boehringer Mannheim) was hybridized overnight to chromosome preparations at 37°C. Following stringency washes (50% formamide at 42°C and 2× SSC at 42°C), the probe was detected using avidin–FITC (Vector Laboratories) and the signal amplified by further incubations with biotinylated anti-avidin (Vector Laboratories) and avidin–FITC. The slides were counterstained with propidium iodide (0.5 µg/ml) and 2.5 µg/ml 4,6-diamidino-2-phenylindole in Vectorshield (Vector Laboratories). Image analysis was performed on a MRC 600 confocal microscope (BioRad).

PCR

The presence or absence of each marker in 87 zebrafish and 48 chicken hybrids cell lines was determined using PCR. For each pair of primers, Mg²⁺ concentration and annealing temperature were determined empirically using a Hybaid thermocycler. PCR conditions were chosen to allow detection of specific products of the donor DNA. PCR products were analysed on 3% agarose gels or on the ABI373 using fluorescently labelled primers.

Primers

The zebrafish-specific primers SSR-2, -9, -13 and -20 were obtained from Research Genetics (MapPairs) (17). Zebrafish cDNAs for Tie1, GATA1, GATA2, TTG2, PU.1, c-myb, embryonic α-globin (hbe1 on LG3), αx-globin (hbx on LG12) and retinoic acid receptor 2β were isolated in the Zon laboratory (M.Thompson, unpublished data). The PCR primers are as follows: Tie1, GGG GAA TTC AAA ATT GCT GAT TTT, CGC GGA TCC ACT CTT GGT GGT GTA; GATA1, GGC CTT TAT TTT AAA CTG, CGC GGA TCC ATT AAC CCT CAC TAA AGG GAG AGC; GATA2, ACA ACC CAG CAT CCC TCC AC, CTC TCG CTT CCA CTT CAT CG; TTG2, CAG CGC AAA ATG CAC CAC, TGC TGT CTT AGC AAG TGT ATC AAA; PU.1, TGC GAA GGT GTT AAT GCA AAG, TAC CAG AAG ATG CCA AGA GCA; c-myb, AAC AAA TCC ATT ATA CTG ATT CAG, TGA TTA TAA ATG GGA AAA ATG; embryonic α-globin, CTG CCA AAG ACA AAG CTG CCG TCA, AGC ATG GAG CTC ACT GAG GTT CAG; αx-globin, CGG CCT GCT GAA CCT

CAG, CAT ATT TGT CAG ACA GAG CC; retinoic acid receptor 2β, GAG GGT CTG GAG GGA GGC, AAA TAA CGC TGA GGC CGA GA. The chicken primer pairs ADL/157, ADL/235 and ADL/236 are available through the WWW at <http://poultry.mph.msu.edu/micronew.html>. The other chicken markers are as follows: Ros-01, TGA AAA GGG ATG GAG TAT GTC A, CCT TGT GAT CTC TCC ACA CTT C; Ros-03, GCA AAG TTA TTC AGG AAC TTG C, AAG TGG TCC CCT GAT TTA ACA; Ros-05, GAA GTG TGG GGT TTG CTG TT, TTG GAT CAG ATG CAT CCA AA; Ros-08, GGA CAA TTC CCC AGT CAC AC, GCA TAT TTG ATT GTG AAA TGG G; Ros-12, GCA ACT GTC ACT GCA AGA TCA, AGG GGT TTC TGA GGG AGT GT; Ros-14, TAC GGT CCT CAK ATT CAT TWC, AMA CAM TGM AAG TGC ATA TCG; Ros-15, GAG TGG AGM GAA CAC CTT TC, CAA CTA GCT GAA ACA GGC ACC.

RESULTS

Generation of whole genome radiation hybrids

We generated our hybrids from the fibroblast-like zebrafish cell line AB9 and the chicken primary fibroblast cell lines F21 and F23. For the two hamster recipient cell lines to which the zebrafish and chicken donor cell lines were fused we chose A23 and Wg3H (14,15). These hamster cell lines are used routinely for human radiation hybrids and allow HAT (hypoxanthine, aminopterin and thymidine) selection for stable hybrids retaining zebrafish or chicken fragments. Fusion conditions were identical to those established for human (10,11), except that the number of cells used in the zebrafish fusion was increased to allow for an expected lower fusion efficiency given the evolutionary distance of the two species. As predicted, we observed that the growth conditions and morphological appearance of the hybrids resembled the recipient hamster cell lines.

The radiation dose applied to break the donor chromosomes is a critical factor, since it influences the mapping resolution of a hybrid panel as well as the retention frequency (i.e. proportion of radiation hybrids that contain donor chromosome fragments) (18). We assessed the effect of radiation dose on retention frequency using four different radiation doses for the chicken hybrids (1000, 3000, 5000 and 10 000 rad). However, no significant difference was detected for the retention frequencies at different radiation doses. This could be due to the small number of hybrids that were examined at each dose. Another factor that affects retention frequency is the utilization of different donor/recipient cell combinations in WGRH fusion experiments. However, we observed no influence of different donor or recipient cell lines on retention frequency (Table 2) in the chicken WGRH.

Characterization of radiation hybrids by PCR

To determine the extent to which donor fragments are retained in our hybrids, several markers were PCR amplified from DNA obtained from the zebrafish and chicken hybrids (4,5,19). Thirteen zebrafish markers were tested on 87 hybrids (chosen at random from a total of 179 hybrid lines) (Table 1). These markers are located on linkage groups III, VI, VIII, XI, XII, XVIII and XXIII (J.Postlethwait, personal communication). The collection of hybrids containing fragments corresponding to all the markers tested here (equal to average retention frequency) was 23.6%. Two markers from the same linkage group (SSR-2 and SSR-20)

were statistically evaluated by the RH2PT computer program (20). These two markers are predicted to be tightly linked on the genetic map. A maximum LOD score of 6.3 with a distance corresponding to 0.41 Ray confirmed that the panel can map markers at small distances. Forty eight chicken radiation hybrids were tested for the presence of 10 microsatellite markers (Tables 2 and 3). The average retention of markers at each of the different radiation doses ranged from 14.5 to 22% (Table 2). Markers from several chicken linkage groups were included in the testing and the average retention frequency for a specific microsatellite marker across all chicken hybrids ranged from 2 to 67% (Table 3). In addition, we observed that the markers derived from microchromosomes have a higher overall retention frequency (17.8%) in the hybrids tested as compared with macrochromosomes (10.6%); three as yet unassigned markers had an average retention frequency of 32%.

Table 1. Summary of markers tested on 87 zebrafish radiation hybrids

Marker ^a	Retention (%)
RARA-2B	10.3
embryonic α -globin, hbe	24.7
Tie1	5.8
GATA1	10.6
GATA2	53.8
α -globin, hbx	54.7
TTG2	24.1
c-myb	20.7
PU.1	27.8
SSR-2	16.1
SSR-20	16.1
SSR-9	12.6
SSR-13	27.6
Average	23.45

^aMarkers used for testing are as indicated. SSR-2, SSR-9, SSR-20 and SSR-13 are microsatellite markers (6,17); the rest are known genes (L.Zon, personal communication).

Table 2. Influence of radiation dose, donor and recipient cell line on retention frequencies of chicken radiation hybrids

Radiation dose (rads)	No. hybrids	Retention (%)	Chicken donor	Recipient
1000	12	22.50	F21	Wg3H-neo
3000	9	17	F23	A23
5000	10	21	F21	Wg3H-neo
5000	9	14.5	F21	A23
10 000	8	16	F21	Wg3H-neo
Total	48			
Average		18.2		

Table 3. Summary of microsatellites tested on 48 chicken radiation hybrids and chromosomal location of the markers

Marker	Linkage group	Chromosome	Retention (%)
ROS-12	E5	4	2
ROS-01	C1	3	29
ADL-157	E6	2	10
ADL-235	E6	2	6
ADL-236	E6	2	6
ROS-05	C40	UN	23
ROS-03	C12	UN	12.5
ROS-08	UN	UN	6
ROS-15	UN	UN	67
ROS-08	UN	UN	23
Average			18.5

Characterization of hybrids by FISH analysis

For additional characterization of our hybrids, fluorescence *in situ* hybridisation (FISH) was used to examine the organization of donor DNA in the hybrids. By hybridizing a probe derived from total genomic DNA from zebrafish or chicken cells to metaphase spreads of the hybrid cell, all hybrids tested retained fragments of the donor DNA. The zebrafish chromosomal fragments were integrated into the hamster genome, attached to the ends of hamster chromosomes or present as distinct 'chromosomes' (Fig. 1a). The chicken hybrids all contained fragments of chicken DNA independently retained as separate 'chromosomes' and fewer fragments integrated into the hamster genome. The extent of donor genome incorporated into the hybrids was determined by reverse painting, i.e. hybridizing fluorescently labelled hybrid DNA onto zebrafish or chicken metaphase spreads. Five zebrafish hybrids were examined and the DNA fragments were all derived from different chromosomes (data not shown). Eighteen chicken hybrids were examined (Fig. 1b) and it appeared that DNA of the microchromosomes was preferentially retained in comparison with DNA fragments of the larger chromosomes.

DISCUSSION

This is the first report describing stable WGRH established by fusing cells from such distantly related species as chicken and zebrafish to hamster. The radiation hybrid panels described in this paper contain a collection of small fragments representing the donor genome. Previously, somatic cell hybrids have been established by fusing chicken erythrocytes with hamster or mouse cells (21,22), chicken microcells with human cells (23) and zebrafish with mouse cells (24). They differ from the results presented in this report in the number and size of the fragments retained in the hybrid cells. In other reports, only a few large donor DNA fragments (up to entire chromosomes) are present in each hybrid, allowing only chromosomal assignments of specific markers or very low resolution maps to be achieved. The utility of the chicken and zebrafish hybrids presented in this report lies in the rapid placement of markers to their exact chromosomal location in the genome. In addition, stable maintenance of the chromosomes in the rodent background as reported previously often required continuous application of the selection scheme.

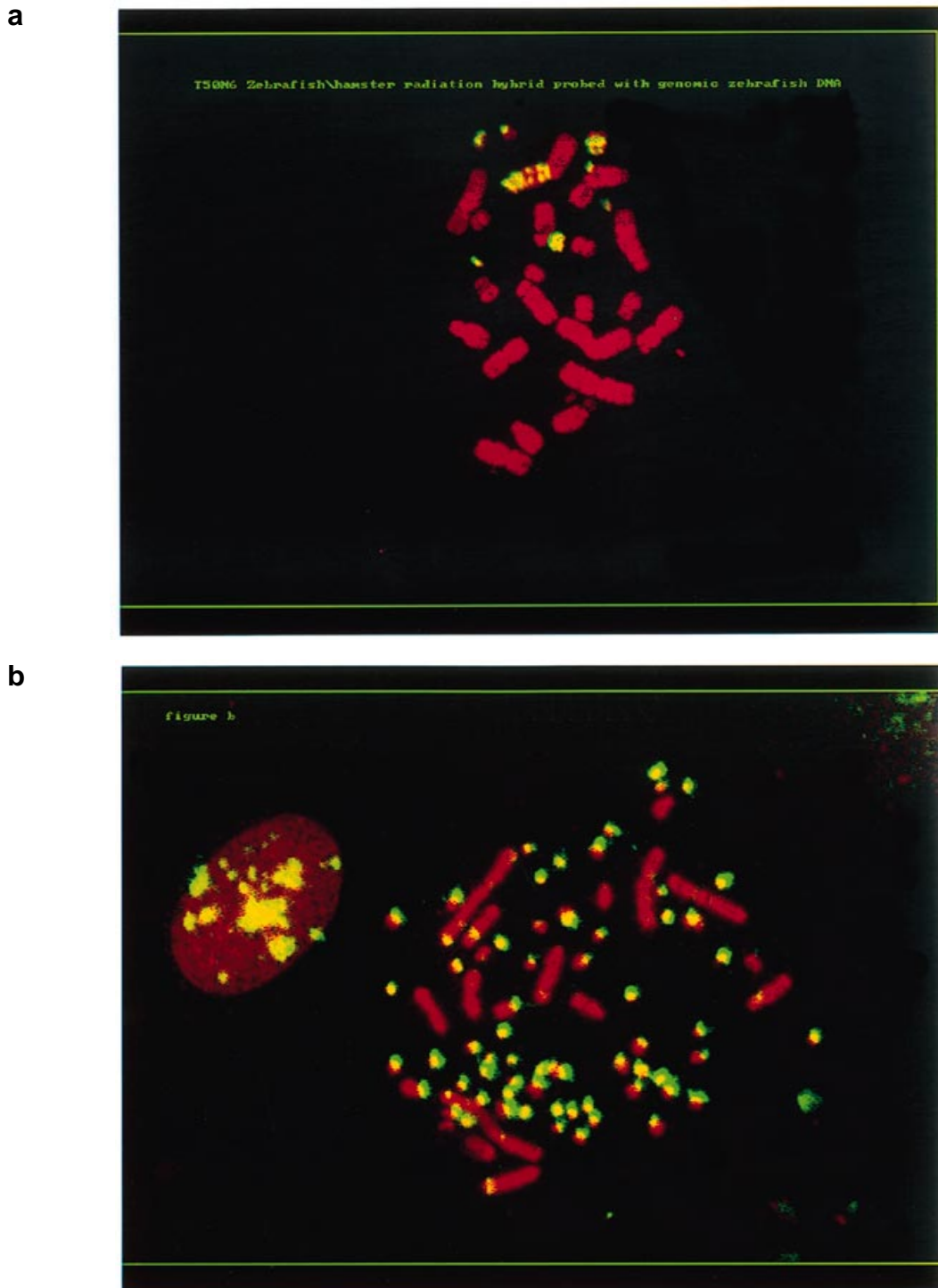


Figure 1. Organization and donor DNA content of radiation hybrids determined by FISH. (a) Metaphase spread of zebrafish hybrid cell line T50N6 hybridized with total genomic zebrafish DNA. (b) Metaphase spread of chicken fibroblasts hybridized with DNA derived from chicken hybrid cell line TFC5.

Our experiments for zebrafish and chicken demonstrate the retention of non-selected chromosomal segments representing several different linkage groups in a hamster background. Chicken as well as zebrafish hybrids were selected for either the hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) or thymidine kinase (*Tk*) genes, but all other fragments have been retained without selection over six (zebrafish) and 25 (chicken)

generations of sub-culture. This allowed us to grow our hybrid over many generations in order to obtain large quantities of DNA for distribution. An apparent preferential retention of microchromosomes in comparison with DNA fragments of the larger chromosomes was observed from hybridizing fluorescently labelled chicken hybrid DNA onto chicken metaphase spreads. This could be attributed to an artefact of preferential hybridization

of DNA to the centromeres of chicken microchromosomes (23). However, as CpG islands appear to be concentrated on chicken microchromosomes (25), the preferential retention of DNA from microchromosomes in our hybrids suggests an even greater utility as a gene mapping resource.

We have shown that it is feasible to create WGRH panels for non-mammalian species and would like to discuss the utility of our panels for extensive mapping. The 'mapping power' of a radiation hybrid panel is influenced by the retention frequency and the radiation dose. The average retention frequencies for both the chicken and zebrafish hybrid panels are comparable with other human and mouse panels provided by our laboratory. In addition, markers distributed throughout the genome have been retained in the panels, indicating a complete representation of genomic fragments. The radiation dose chosen for the zebrafish panel has proven useful for human and mouse panels and will provide resolution in the range 1–10 cM. We conclude that the basic characteristics of the panels presented in this report are comparable with the already extensively used mapping panels for human and mouse. We recommend using the same dose for the chicken panel. A pair of tightly linked markers (SSR-2 and SSR-20, unresolved on the genetic map) was chosen to evaluate the 'mapping power' of the zebrafish whole genome radiation hybrid panel (5,26). Statistical analysis using the RH2PT computer program (27) gave a maximum LOD score of 6.30 for linkage between SSR-2 and SSR-20 (distance = 0.402 Ray). In order to generate robust radiation hybrid maps, at least one break must be observed between a pair of adjacent markers. Since the retention pattern of SSR-2 and SSR-20 observed in the subset of 87 radiation hybrids was similar but not identical (probability of breakage = 0.331), a higher resolution map can be achieved compared with conventional mapping methods using this panel of zebrafish radiation hybrids. The organization of the donor DNA fragments in our chicken and zebrafish hybrids is similar to radiation hybrids for other organisms, especially human and mouse (10,11). This shows that donor DNA fragments do not have to be integrated into the hamster genome in order to be retained in hybrid cells. Based on these characteristics and comparisons with currently available and proven mapping panels, we conclude that the zebrafish panel described in this report will have great utility for fish mapping projects. Given a retention frequency of ~20%, a panel of 100–200 hybrids will be sufficient for most mapping purposes (20). Other strategies for increasing the power of hybrid panels include generating additional hybrids and pooling them to increase retention frequencies (28). For genome-wide chicken radiation hybrid mapping we will have to generate additional hybrids; the currently available hybrids, however, will be sufficient for localized mapping projects. A larger number of additional hybrids (>400) can easily be generated, since fusion efficiencies for zebrafish and chicken were relatively high, $0.5-1 \times 10^{-6}$ and $2-9 \times 10^{-6}$ respectively.

Radiation hybrid mapping does not require extensive searches for polymorphisms in the genes to be mapped, since it utilizes the molecular differences between the donor and the recipient cell lines. In addition, this methodology does not depend on any crosses. Such a mapping procedure is easily amenable for high throughput analysis and the required map location will be obtained at very high resolution. The reported WGRH panels will be an invaluable resource for developing integrated genome maps and will facilitate gene mapping/finding in many other non-mammalian vertebrates with currently limited genomic information. All hybrid panels will be distributed through Research Genetics or by contacting the authors directly.

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