Molecular cytogenetic characterization of a human thyroid cancer cell line

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ABSTRACT

The incidence of papillary thyroid carcinoma (PTC) increases significantly after exposure of the head and neck region to ionizing radiation, yet we know neither the steps involved in malignant transformation of thyroid epithelium nor the specific carcinogenic mode of action of radiation. Such increased tumor frequency became most evident in children after the 1986 nuclear accident in Chernobyl, Ukraine. In the twelve years following the accident, the average incidence of childhood PTCs (chPTC) increased over one hundred-fold compared to the rate of about 1 tumor incidence per 10^6 children per year prior to 1986. To study the etiology of radiation-induced thyroid cancer, we formed an international consortium to investigate chromosomal changes and altered gene expression in cases of post-Chernobyl chPTC. Our approach is based on karyotyping of primary cultures established from chPTC specimens, establishment of cell lines and studies of genotype-phenotype relationships through high resolution chromosome analysis, DNA/cDNA micro-array studies, and mouse xenografts that test for tumorigenicity. Here, we report the application of fluorescence in situ hybridization (FISH)-based techniques for the molecular cytogenetic characterization of a highly tumorigenic chPTC cell line, S48TK, and its subclones. Using chromosome 9 rearrangements as an example, we describe a new approach termed 'BAC-FISH' to rapidly delineate chromosomal breakpoints, an important step towards a better understanding of the formation of translocations and their functional consequences.

Keywords: Thyroid cancer, Chernobyl, radiation effects, cytogenetics, structural chromosome aberrations, fluorescence *in situ* hybridization, spectral karyotyping, chromosome 9.

INTRODUCTION

Thyroid cancer (TC) is a malignant disease of the thyroid gland in which epithelial cells spontaneously proliferate, disregulate the production of essential thyroid hormones, and undergo apparently irreversible changes in their repertoire of expressed genes. More advanced tumors often develop the ability to break out of the thyroid capsule, infiltrate the nearby lymphatic system, and eventually form distal tumors in bone and soft tissues. While not a major cause of cancer death in the U.S. (or elsewhere), unrecognized and aggressively growing, metastasizing cancers carry an annual death toll that approaches the total number of lives lost due to automobile accidents in all of the 50 U.S. states combined (Chen and Udelsman, 1998). How is it possible that the rate of thyroid tumor death decreased less than marginal in the last 30 years? The most likely answer lies in the complexity of the disease and the biological breadth of TC phenotypes and their yet to be explored underlying genotypes.

Release of radioactive ^{131}I in the aftermath of the Chernobyl nuclear power plant accident in April 1986 led to an unprecedented exposure of thyroid tissues in inhabitants of the nearby regions of Belarus, Ukraine and Russia. Normally, iodine is introduced through the diet and rapidly absorbed in the intestine as inorganic iodide. Serum iodide is concentrated by the thyroid gland through an active transport mechanism and incorporated in the protein thyroglobulin to form iodotyrosines, the precursors of thyroid hormones (Nagataki and Ingbar, 1991). As early as four years after the accident, the incidence of childhood papillary thyroid carcinomas (chPTC) had increased drastically exceeding 10 cases per million children per year in the most contaminated Gomel region. Here, rates of 0.5 tumors per million children per year were recorded prior to the accident (Baverstock et al. 1992; Kazakov et al., 1992; Williams et al., 1993). This rate subsequently increased reaching almost 100 cases/million children/year in 1994 (Gembicki et al., 1997).

The papillary variant of TC (PTC) is the most common form of this neoplasia accounting for about 80% of sporadic cases of TC (Jossart and Clark, 1994) with some of the shared or

common alterations being identified. Although very rare in children, PTC is the most common primary cancer of the thyroid gland (Chang, 1990). Even though it usually pursues an indolent course, more aggressive tumors have been observed in some adult cases and, in particular, among the childhood cases from Belarus (Fugazzola et al., 1995; Nikiforov et al., 1995). Rearrangements of chromosomes 1 and 10 are frequently observed cytogenetic changes in PTC (Bongarzone et al., 1989, 1993; Herrmann et al., 1991a; Jossart et al. 1995), while loss of heterozygosity (LOH) for several loci on chromosome 3 had initially been reported only in follicular TC (Herrmann et al., 1991b). Adult PTC studies revealed clonal structural chromosomal abnormalities involving chromosome 10q11. A correlation of alterations involving the chromosomal region 10q11 to thyroid tumorigenesis was supported by the frequent finding of a truncated ret proto-oncogene, which localizes to the same chromosomal band (Pierotti et al., 1992). Reported aberrations range from unconfirmed small interstitial deletions to larger intra- and interchromosomal rearrangements and chromosome loss. Ward et al. have reported LOH in PTC for loci on chromosomes 3, 10, and 11 (Ward et al., 1998). While cytogenetic studies have reported certain changes in cases of adult PTC, there is little information regarding the cytogenetic alterations in chPTC (Zitzelsberger et al., 1999).

Here, we describe the characterization of derivative chromosomes in the chPTC line S48TK and its subclones based on Spectral Karyotyping (SKY) hybridization and chromosome-specific probes analyses. SKY is a powerful technique that reveals chromosomal exchanges that might have remained undetected by G-banding. SKY is based on the replacement of standard emission filters in a fluorescence microscope with an interferometer to record high-resolution spectra from fluorescently stained specimens. This bio-imaging system combines the techniques of fluorescence optical microscopy, charged coupled device imaging, Fourier spectroscopy, and software for digital image analysis. The power of this technology has been demonstrated by specific FISH painting of all 24 human chromosomes in metaphase spreads (Garini et al., 1996; Schröck et al., 1996). SKY has been applied in cancer studies (Schröck et

al., 1996; Zitzelsberger et al., 1999, 2001), prenatal diagnosis (Ning et al., 1999), and human oocytes and polar bodies (Márquez et al., 1998; Willadsen et al., 1999). Our group pioneered the development of Spectral Imaging protocols for simultaneous enumeration of 10 chromosomes in interphase nuclei using Spectral Imaging (SIm) (Fung et al., 1998, 2000), and applied it to the aneuploidy screening for human preimplantation embryos. In the studies described here, we applied SKY for initial screening of S48TK-derived metaphase spreads following G-banding analysis. Due to the large number structural chromosome aberrations found, we followed up with hybridization of individual whole chromosome painting (WCP) probes and a bacterial artificial chromosome (BAC)-based hybridization scheme termed 'BAC-FISH'. Currently, the BAC-FISH assays can be used to stain the entire euchromatic portion of chromosomes, individual chromosome arms and single or multiple chromosome bands. In the present communication, we summarize our approaches and findings with respect to chromosome aberrations in S48TK clones involving DNA derived from chromosome 9.

MATERIALS AND METHODS

Cell cultures and preparation of metaphase spreads

Control metaphase spreads were made from phytohemagglutinin-stimulated short-term cultures of normal male lymphocytes according to the procedure described by Harper and Saunders (Harper and Saunders 1981). Fixed lymphocytes were dropped on ethanol-cleaned slides in a CDS-5 Cytogenetic Drying Chamber (Thermatron Industries, Inc., Holland, MI) at 25°C and 45-50% relative humidity.

The S48TK cultures were established as described by Zitzelsberger et al. (1999). All procedures followed protocols approved by the Ethics Committee of the Bavarian Board of Physicians and the LBNL/UC Berkeley Committee on Human Research regarding use of surplus surgical tissues for research. Briefly, S48TK lines were obtained form the tumor tissue of a 14 year old patient (7 years at time of exposure to elevated levels of radiation) undergoing surgery the Center for Thyroid Tumors in Minsk, Belarus, following the diagnosis of Hashimoto's thyroiditis and PTC. Initial chromosome preparations were carried out after an *in vitro* culture of cells for 8–21 days. Later on, clones were isolated by limiting dilution and cultured for more than 20 passages. The clone number is indicated following the name of the primary line, S48TK. For example, S48TK18 refers to clones 18 of cell line S48TK. After G-banding with Wright's staining solution, karyotyping was performed according to the International System for Human metaphase chromosomes were prepared after in vitro culture of S48TK cells for 8 to 21 days. After G-banding with Wright's staining solution, karyotypes were recorded according to the International System for Human Cytogenetic Nomenclature (Mitelman, 1995).

Preparation of DNA probes

Our procedures for preparation of DNA probes from BAC/PAC clones (Shizuya et al., 1992; Ioannou et al., 1994) have been described in detail before (Weier et al., 1995, 2006). Prior to the chromosome 9-specific FISH studies, 150 BAC clones from the Sanger Center 1

Mbp set were re-arrayed on two 96-well microtiter plates. Individual clones were arranged so that the entire chromosome 9-specific clone set was contained in 15 rows termed 'pools', with 8-12 clones per pool in individual row positions. This created pools '9-1' to '9-15', each of which covers several megabase pairs (Mb) of DNA on chromosome 9 (Table I). Pools 9-1 to 9-5 and pools 9-6 to 9-15 map to the short and long arm of chromosome 9, respectively. When large numbers of clones were grown, cultures were done in 2ml of medium in 96 deep well plates (Beckman, City of Hope, CA). Individual clones were grown overnight in 8-20 ml of Luria broth (LB) medium (Maniatis et al., 1986) containing 12.5 μg/ml chloramphenicol (Sigma, St. Louis, MO) and the DNA was extracted using an alkaline lysis protocol (Birnboim and Doly, 1979; Weier et al., 1995). For preparation of DNA pools or 'super-pools', i.e., combination of 2 or more pools, clones were grown individually and pooled prior to DNA extraction. Quality control and quantification of the DNA was typically done by agarose gel electrophoresis and fluorometry, respectively.

All DNA probes were prepared by random priming (BioPrime kit, Invitrogen, Gaithersburg, MD) incorporating biotin-14-dCTP (part of the BioPrime kit), digoxigenin-11-dUTP (Roche Molecular Biochemicals, Indianapolis, IN), fluorescein-12-dUTP (Roche Molecular Biochemicals), Cy5-dUTP (Amersham, Arlington Heights, IN) or Cy5.5-dCTP (Perkin Elmer, Wellesley, MA) (Fung et al., 2001a,b). Between 0.5 μl and 3 μl of each probe along with of 4 μl human COT1™ DNA (1 mg/ml, Invitrogen) and 1 μl salmon sperm DNA (20 mg/ml, 3'-5', Boulder, CO) were precipitated with 1 μl glycogen (Roche Molecular Biochemicals, 1 mg/ml) and 1/10 volume of 3M sodium acetate in 2 volumes of 2-propanol, air dried and resuspended in 3 μl water, before 7 μl of hybridization master mix (78.6% FA, 14.3% dextran sulfate in 2.9x SSC, pH 7.0) were added. This gave a total hybridization mixture of 10 μl.

In the following, we will refer to the combination of all 150 BAC-derived DNA probes as whole chromosome painting probe (WCP), and call combinations of pools 9.1-9.5 and 9.6-9.15 'chromosome arm probes (CAP)' for chromosome 9p and 9q, respectively. To investigate

chromosome 9 rearrangements in S48 with higher resolution, we labeled DNA extracted from 9p-specific clones and pairs of chr.9q-specific, adjacent pools with 5 different fluorochromes, and refer to these probes as 'chromosomal rainbow probes (CRP) (Table1).

Comparative Genomic Hybridizations

Comparative genomic hybridization (CGH) with DNA isolated from the primary culture as well as cell lines established from case S48TK was performed following standard procedures as described for a case S42 (Kallioniemi et al., 1992; Lehmann et al., 1997). Briefly, genomic DNA was isolated from the primary culture as well as from cell lines and labeled with biotin-16-dUTP (Roche Molecular). Normal female reference DNA was isolated from peripheral lymphocytes of a healthy donor and labeled with digoxigenin-11-dUTP (dig-11-dUTP). After hybridization to normal metaphase spreads of a healthy donor, labeled DNA probes were detected with streptavidin-Cy2 or avidin DCS-FITC (Vector Inc., Burlingame, CA) and anti-digoxigenin-Cy3/rhodamin conjugates. Slides were counterstained with DAPI for chromosome identification. For CGH analysis, eight or more metaphases were analyzed. Averaged profiles were generated by CGH analysis software (MetaSystems, Altlussheim, FRG or Vysis, Downers Grove, IL) from 10-15 homologous chromosomes and interpreted according to published criteria (Kallioniemi et al., 1994; Isola et al., 1995).

SKY analysis

Spectral karyotyping is a molecular cytogenetic procedure to screen the entire human genome for interchromosomal translocations by hybridization of 24 different WCP probes mixtures to metaphase spreads. We applied SKY to case S48TK and its subclone S48TK18, and identified its complex aberration patterns (Zitzelsberger et al., 1999). SKY analyses followed essentially the recommendations of the manufacturer of the reagents and the SKY imaging instrumentation (Applied Spectral Imaging (ASI), Carlsbad, CA). Briefly, fixed cells on slides were pretreated with 50 µg/ml pepsin (Amresco, Solon, OH) in 0.01N HCl for 5-13 min at

37°C before immersion in phosphate buffered saline (PBS) for 5 min. Next, the slides were incubated in 1% PFA in PBS for 5 min, then in PBS for 5 min. After a immersion in a 70%, 80%, 100% ethanol series (2 min per step), the slides were air dried. Cells on slides were denatured for 5 min at 76°C in 70% formamide (FA)(Invitrogen, Gaithersburg, MD)/2× SSC and then dehydrated in 70%, 80%, and 100% ethanol (2 min per step) before air drying. Meanwhile, the hybridization mixture (ASI) containing 24 painting probes, each specific for one human chromosome type and labeled with combinations of five different reporter molecules was denatured for 5-6 min at 76°C, and pre-annealed/-blocked for 30-90 min at 37°C. The pre-blocked hybridization mixture was then applied to each slide, cover slips were place on top and sealed with rubber cement. The hybridization reaction proceeded for 18-42 h at 37°C. After hybridization, all slides were washed three times (10 min each time at 43°C) in 50% FA/2× SSC, then twice in 2× SSC (10 min each time at 43°C). The slides were mounted with 8 µl of 4′,6-diamidino-2-phenylindole (DAPI, 0.5 µg/ml, Calbiochem, La Jolla, CA) dissolved in anti-fade medium (Fung et al., 2001a,b). Metaphases images were acquired with the Spectracube system (ASI) and analyzed with the SKYVIEW software (Veldman et al., 1997; Garini et al., 1996).

RESULTS AND DISCUSSION

G-banding analysis of chPTC case S48TK

The analysis of case S48TK revealed a near triploid karyotype with numerous complex chromosomal rearrangements. However, G-banding is not very specific, and the tumors might carry far more translocations than revealed in banding studies. On the other hand, the G-banding analysis indicated several chromosomal regions involved in translocations. Further refinement of breakpoint locations and the correct identification of translocation and marker chromosomes required much more specific, high-resolution techniques.

Comparative Genomic Hybridization

CGH revealed differences between individual clones of line S48TK as well as structural aberrations, gains and losses of DNA and indications the approximate positions of chromosomal breakpoints. Bound DNA derived from S48 clone 20 was detected with avidin-FITC (green), and bound probe derived from S48 clone 37 was detected with rhodamine-labeled antibodies (red). Thus, chromosomal areas appearing in red in Fig. 2A are present in more copies in clone 37, while the green areas have a higher copy number in clone 20. Strong yellow fluorescence indicates extra copies in both clones. Color changes along the chromatids indicate the presence of breakpoints. The chromosomes 1, 2, 7, and 9 are indicated in the figure. We also noted a very well delineated interstitial deletion on the long arm of chromosome 13 present in both clones. Although CGH analysis detected losses on 1q and 13q as well as gains on 1p, 2p and 22q, we focused our investigation on the structural alterations leading to overrepresentation of the pericentromeric region of chromosome 9 and the proximal long arm of chromosome 9q since such alteration have not been described previously. Figure 2B shows the typical CGH profiles observed with S48TK DNA (red) hybridized in combination with normal control DNA (green) to metaphase spreads prepared from a normal individual. lthough the inclusion of unlabeled COT1 DNA in the hybridization mixture lead to a virtually complete block of

hybridization to the α-satellite DNA containing pericentromeric region and the satellite III DNA cluster on the proximal long arm, the results suggest high level amplification of a large region extending from the proximal p-arm to about1/2 of the q-arm.

SKY Analyses of S48TK clones

The DAPI image was used to identify chromosomes. Chromosomes in the hybridization image were assigned 'classification colors', which link the fluorescence spectra to chromosome-specific probe mixture (Veldman et al., 1997; Garini et al., 1996). G-banding analysis had demonstrated a near triploid karyotype of S48, and SKY confirmed the presence of several marker chromosomes in this childhood PTC (Zitzelsberger et al., 1999). Most rearrangements seemed to have led to loss of genetic material, and we found only a single reciprocal translocation, a t(1;4). While the identification of marker chromosomes based on G-banding contained several errors, the banding analysis suggested specific autosomes such as chromosome 1, 2, 8-10 and 19 to be involved in rearrangements. The translocation t(1;6) identified by SKY analysis in case S48 (Fig.3A) is of particular interest, because unpublished studies performed independently in our laboratory on the irradiated tumorigenic thyroid epithelial cell line HTG1 (Riches at al., 1994, 1997) revealed a similar translocation. SKY analysis of case S48 also demonstrated the power of Spectral Karyotyping compared to G-banding analysis. A single SKY hybridization experiment was sufficient to characterize translocation products and identify marker chromosomes. This information could not be generated by G-banding (Zitzelsberger et al., 1999).

Analysis of cell line S48TK had indicated some heterogeneity, although all metaphases examined showed some of the same marker chromosomes, such as the der(7;9;15). We therefore subcloned S48 by limiting dilution. The subclone S48TK18 was analyzed in detail using SKY, and revealed a small translocation of chromosome 9 DNA into a der(8;15)(arrows in Figs.3B,C). Figure 3 B shows the partial SKY analysis of S48TK18, and the small insertion

(arrow). Since S48TK18 produces rapidly growing tumors in nude mice (data not shown), and rearrangements of chromosome 9 in radiation–induced chPTC's have not been described previously, we focused the present study on the characterization of chromosome 9 derived fragments and, in particular, on determining the origin of the previously undetected chromosome 9-specific DNA translocated to the der(8;15). While the SKY analysis of S48TK18 produced results impossible to obtain with either banding or CGH analysis, SKY did not allow to cytogenetically map the translocated fragments or translocation breakpoints. We therefore applied high resolution BAC-FISH in the analysis of the der(8;15).

BAC-FISH assays with predetermined levels of resolution

We applied BAC clones from the Sanger Center (Cambridge, UK) 1Mbp collection (Fiegler et al., 2003) for characterization of chromosome 9-derived DNA translocated in S48TK18 (S48TK18). The results of these experiments are summarized in Fig.3D-H. Prior to FISH experiments, we re-arrayed 150 chromosome 9–specific BAC clones from the collection as described in the Material and Methods section. The new 96-well plate outline places 15 pools of 8-12 individual clones into individual rows of two microtiter plates so that the effects of differential growth are minimized by growing clones individually and pooling them by row prior to DNA extraction.

Initially, we tested the specificity and complexity of the BAC pools by hybridizing WCP and CAP probes. Representative images are shown in Fig.3D-F. The results reconfirmed mapping results from the Sanger Center: 50 clones mapped exclusively to chromosome 9p, while 100 clones mapped to chromosome 9q, as predicted (Fig.3E, Table I). The 150 BAC clones could also be combined and labeled with the same reporter molecule to result in a WCP9 probe (Fig.3D). This WCP9 probe mixture was used initially to localize chr.9-derived material in S48TK18 metaphase cells (Fig.3D) revealing four different types of chromosomes carrying chr.9 DNA. When hybridized to metaphase spreads prepared from cell line S48TK18, the CAP9

probe mixtures demonstrated that only one of the four before mentioned chromosome types carried DNA from chr. 9p (Fig.3E). We also noted some variability: the metaphase spread shown in Fig.3F, for example, showed two copies of this (9p+9q; red+green)-chromosome, while some metaphase spreads showed only one copy.

In the studies presented here, our main interest focused on the BAC-FISH-based delineation of small, often called 'cryptic' translocations. In particular, we were interested in determining the origin of the small piece of chr.9 DNA inserted in the der(8;15)(Fig.3F, arrow). To test the feasibility of hybridizing either individual pools or adjacent pairs of pools, we prepared a number of CRP9 sets as listed in Table 1. Figure 3G shows, for illustration purposes, an overlay of some of the hybridization result images captured with a filter based imaging system, in which rhodamine/Spectrum Orange and Texas Red signals as well as Cy5 and Cy5.5 signals appear in the same channels.

Successive BAC-FISH hybridizations with increasing resolution (WCP9 – CAP9 – CRP9) allowed us to determine the regions represented by pools 9-10 and 9-11 as source of this extra material. Although not too clearly visible in Fig.3H, the signals on the der(8;15) were caused by pool 9-10 (green) as well as a fraction of pool 9-11 (red) probes. Thus, four overnight BAC-FISH hybridizations allowed to narrow the size and origin of the translocated fragment to an interval around 96 Mb - 109 Mb on chromosome 9 (Table 1), an interval that will be further refined by multicolor hybridization of individual BAC probes.

In summary, the studies presented here on a case of post-Chernobyl chPTC emphasize the need for sensitive, high resolution karyotyping techniques. While screening procedures such as G-banding, SKY and CGH have provided important leads to the investigations of structural chromosome aberrations in S48TK cells, the BAC-FISH assays lead to rapid delineation of the breakpoints. Thus, the BAC reagents recently prepared and distributed by the Sanger Center, UK, with support from the WellcomeTrust, UK, proved to be of great value in the molecular cytogenetic analysis of thyroid tumor cells.

Our interest in mapping breakpoints in chPTC benefits much from the available, mapped BAC clones. Eventually, these investigations will help us to understand the mechanisms that lead from radiation damage to DNA (such as DNA strand breaks or adducts) or to the intracellular environment (via generation of reactive radical groups) to the formation of translocation chromosomes. Once a breakpoint region and a co-linear BAC clone have been identified, DNA sequence analysis will help to identify common facilitators of translocations such as DNA repeats, ALU sequences, in particular (Babcock et al., 2003), or other kinkable DNA elements (Mashkova et al., 2001).

The studies presented here provide a roadmap that can be applied in a multitude of studies to delineate translocation breakpoints in one or two weeks, and through DNA database analyses, identify genes in the proximity of breakpoints and critical DNA elements at the junctions. This will help to better understand the cytogenetic events and alterations leading to malignant transformation of cells and eventually, to neoplastic disease and tumors.

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Table I. BAC Pool Positions, Sizes, and Labels [a]

Pool	Start [bp]	Stop [bp]	Size [Mbp]	WCP label	CAP label	CRP label	Pools
9-1	991152	7418276	6.43	biotin	digoxigenin	FITC	-
9-2	7267081	14586697	7.32	biotin	digoxigenin	Sp.Orange	-
9-3	15219945	22579721	7.36	biotin	digoxigenin	Cy5	-
9-4	23376562	29639053	6.26	biotin	digoxigenin	TR	-
9-5	30199650	43076412	12.88	biotin	digoxigenin	Cy5.5	-
9-6	68358409	76253493	7.90	biotin	biotin	Sp.Orange	-
9-7	76861448	83525574	6.66	biotin	biotin	Sp.Orange	-
9-8	84220295	88066130	3.85	biotin	biotin	Cy5	-
9-9	88644066	95598231	6.95	biotin	biotin	Cy5	-
9-10	96060259	100197864	4.14	biotin	biotin	FITC	avidin
9-11	100788649	109360971	8.57	biotin	biotin	FITC	digoxigenin
9-12	109953950	115297394	5.34	biotin	biotin	TR	-
9-13	115288454	121178266	5.89	biotin	biotin	TR	-
9-14	121691418	130045763	8.35	biotin	biotin	Cy5.5	-
9-15	130510169	138274031	7.76	biotin	biotin	Cy5.5	-

[a] Probes labeled with biotin or digoxigenin were detected with avidin-FITC and rhodamine-conjugated antibodies against digoxigenin, respectively. [Abbreviations: WCP: whole chromosome painting probe, CAP: chromosome arm probe, CRP: chromosomal rainbow probe.]

Figure Legends

Figure 1. G-banded karyotype of the chPTC cell line S48TK.

Figure 2. CGH Analysis.

A) The CGH analysis of S48TK clones revealed several chromosomes with structural aberrations. In this figure showing the results of hybridizing one clone against another, chromosomes 1, 2, 7, 9, and 13 are indicated. The present study focused on alteration involving chromosome 9-derived genetic material.

B) CHG profiles (red:green ratio along the chromosome) for the homologues of chromosome 9. Left: the region of extra copies is indicated by a green bar left to the chromosome 9 ideogram. Right: The CGH profiles (red lines) for the 2 copies of chromosome 9 show strong amplification of the proximal long arm when S48TK DNA was hybridized to normal metaphase spreads in the presence of labeled normal DNA. The blue line represents the mean.

Figure 3. Fluorescence *in situ* hybridization analysis of chromosomal rearrangements in S48TK. (A-B) Results of SKY analyses. The arrow in B points to a small insertion of chromosome 9 material into a der(8;15) in clone S48TK18, which had been overlooked initially. (C) An enlarged picture of the various chromosomes carrying chromosome 9-derived material. The arrow points to the small amount of inserted chromosome 9 material. (D) Development of probe mixtures for BAC-FISH. (D) Hybridization of WCP probes comprised of 150 BAC-derived probes to normal homologues of chromosome 9 (top) and metaphase chromosomes from S48TK18. (E-F) Analysis of metaphase spreads using chromosome arm-specific probes (CAP9) revealed only a single derivative chromosome type with material derived from chromosome 9p in clone S48TK18 (bottom). The hybridization pattern on normal chromosomes 9 are shown on top. (F) FISH results with CAP9 using S48TK18 metaphase spreads. The schematics on the left in D-F illustrate the anticipated FISH results. (G) Hybridization of CRP9 to normal homologues of

chromosome 9. See text for details. (H) An image of a partial metaphase from S48TK18 hybridized with probes from pool9-10 (green) and 9-11 (red) shows signals (arrow) from both pools in the region of interest on the der (7;15).