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*Cover:* Brittany-Beagle dog with hereditary canine spinal muscular atrophy (see Cork *et al.*, pp. 69–76). Photograph courtesy of Mr. Victor Raspa.



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# Methods in Laboratory Investigation

## Interphase Cytogenetics in Paraffin Embedded Sections from Human Testicular Germ Cell Tumor Xenografts and in Corresponding Cultured Cells

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**A protocol was developed that allows determination of chromosome aberrations in interphase nuclei from paraffin embedded human tissues. As a model system tissue sections from xenografts derived from three testicular germ cell tumors (two teratocarcinoma and one embryonal carcinoma), as well as sections from normal intestine and testicular tissues, were hybridized with a biotin-labeled DNA probe specific for 1q12. For comparison, isolated nuclei in suspension and metaphase spreads from *in vitro* cell lines derived from the respective xenografts were assayed. Our results show that interphase tumor cells with aberrant numbers of chromosome 1 can be reliably detected both *in vitro* and *in vivo*. Subclones with two and three labeled chromosomes, respectively, could be defined and quantitatively evaluated. Present and future possibilities to pinpoint specific chromosome aberrations directly in cells present in body fluids or in tumor tissues are discussed.**

**Additional key words: *In situ* hybridization, chromosome 1 aberrations.**

Undifferentiated forms of malignant tumors present problems in diagnosis and consequently often in prognostic and treatment modalities. This is especially true for human testicular germ cell tumors (16). Biochemical markers defined so far, such as α-feto-protein (33), human placental-like alkaline phosphatase (13), and β-human chorionic gonadotropin are not always expressed consistently (41). It is therefore important to define additional and more stable tumor markers with diagnostic and/or prognostic value. Chromosomal markers might be particularly useful in this respect (8, 35). Conventional chromosome banding studies, however necessitate *in vitro* cultivation of tumor cells. In short-term cultures the number and quality of metaphase spreads is often insufficient. In long-term cultures certain cells may gain a selective advantage and become predominant. Furthermore, additional chromosome aberrations may arise during long term *in vitro* cultivation. Thus for various reasons in both short- and long-term cultures *in vitro* it is unclear whether the cells available for karyotyping are representative for the tumor cell population *in vivo*.

Recent visualization of individual chromosomes and chromosomal segments within the interphase nucleus of various human cultured cell types has become possible by nonradioactive *in situ* hybridization of chromosome specific DNA probes (5–7, 12, 17, 26, 27, 37, 40). The usefulness of this approach, which has been termed interphase cytogenetics (5), in studies of malignant solid tumors was demonstrated by the detection of numerical and structural aberrations of specific chromosomes in nuclei from *in vitro* cultivated neuroectodermal tumor cell lines and from solid bladder tumors (6, 7, 17). In the present paper we describe the adaptation of this new approach to routine paraffin embedded tissue sections that is indispensable for the more general application of interphase cytogenetics in histopathology. We also describe a simple protocol for the *in situ* hybridization of isolated nuclei in suspension (40). Compared with cells hybridized on slides this approach results in an improved hybridization efficiency and may be particularly useful to study chromosome aberrations in cells from body fluids, including semen. As a model system we have assessed the ploidy of chromosome 1 in interphase nuclei from both normal and germ cell tumor tissue sections. Chromosome 1 frequently presents numerical and/or structural aberrations in these tumors (1, 8, 35).

## EXPERIMENTAL DESIGN

A protocol is described, which yields optimum results with sections from germ cell tumor xenografts and intes-

tinal tissue after *in situ* hybridization with a biotinylated DNA probe, specific for 1q12. For this purpose tumors from two patients with teratocarcinoma and one patient with an embryonal carcinoma were subcutaneously grown in NMRI nude mice. Tumor transplants and tissues from testis and human small intestine were fixed with four different routine protocols, embedded in paraffin and sectioned at a thickness of 4, 6, and 8 μm. For *in situ* hybridization on these sections various parameters such as tissue attachment, permeabilization, and denaturation were tested. For comparison, cell cultures were established from xenografts of the three tumors. Metaphase spreads and interphase cells fixed on slides, as well as isolated nuclei in suspension prepared from these cell lines were also hybridized *in situ*. Visualization of hybridized probe was performed by a peroxidase reaction or by an immunohistochemical fluorescein isothiocyanate-conjugated antibody reaction.

## RESULTS AND DISCUSSION

*In situ* hybridization with the biotinylated probe pUC1.77 was performed with human testicular germ cell tumors transplanted into nude mice and embedded directly for paraffin tissue sections or further cultivated *in vitro*. Stringent hybridization conditions were employed as previously described, which allow the hybridization of pUC1.77 specifically to the C band 1q12. Minor hybridization sites to the centromeric regions of other chromosomes were negligible under these conditions (7, 12, 17).

TABLE 1. EVALUATION OF HYBRIDIZATION SIGNALS AFTER *IN SITU* HYBRIDIZATION WITH BIOTINYLATED PROBE pUC1.77 (PEROXIDASE DETECTION) IN THREE DIFFERENT GERM CELL TUMORS AND THEIR CORRESPONDING *IN VITRO* CELL LINES

| Cell type | Percentage of nuclei with spot number | | | | | | Mean | *N* |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | | |
| Germa 1 | | | | | | | | |
| Metaphases | | 10 | **86** | 1 | | 3 | 3.00 | 71 |
| Nuclei on slides | 5 | 18 | **66** | 5 | 2 | 4 | 2.92 | 175 |
| Nuclei in suspension | | 15 | **78** | 1 | | 6 | 3.04 | 160 |
| Tissue sections | | 34 | **65** | | | 1 | 2.72 | 60 |
| Germa 2 | | | | | | | | |
| Metaphases | | 20 | **67** | 8 | | 4 | 2.98 | 53 |
| Nuclei on slides | 3 | 26 | **60** | 5 | 1 | 5 | 2.90 | 201 |
| Nuclei in suspension | | 16 | **65** | 3 | | 15 | 3.31 | 198 |
| Tissue sections | | 10 | **75** | 14 | | 1 | 3.10 | 63 |
| Germa 3 | | | | | | | | |
| Nuclei in suspension | | **62** | 37 | 1 | | | 2.38 | 132 |
| Tissue sections | 3 | 25 | **70** | 2 | | | 2.69 | 63 |
| Normal lymphocyte | | | | | | | | |
| Nuclei in suspension | 2 | **98** | | [a] | | | 1.99 | 238 |
| Normal human tissues | | | | | | | | |
| Small intestine | 13 | **86** | | | | | 1.87 | 62 |
| Testis | **62** | 38 | | [a] | | | 1.38 | 196 |

Numbers of hybridization experiments were two for metaphases and nuclei on slides, one for nuclei in suspension, and at least two for sections of each tumor and normal tissue, respectively. Peak fractions are presented in bold characters. *N*, number of evaluated nuclei. For statistical evaluation of the data see text.

[a] One nucleus with four hybridization signals was observed.

### *IN SITU* DELINEATION OF 1Q12 *IN VITRO* CULTIVATED CELLS

Metaphase Spreads. After *in situ* hybridization with probe pUC1.77 metaphase spreads from cell lines Germa 1 and 2 showed predominantly three hybridization signals (Table 1). Additional G-banding studies further confirmed trisomy for the 1q12 region in these cell lines (data not shown). In Germa 1 the labeled chromosomes did not show any obvious structural aberrations. One or two labeled marker chromosomes with a partial deletion of 1q (1q2 → 1ter) were observed in Germa 2 in addition to apparently normal chromosomes 1 (Fig. 1*b*, Table 2). From Germa 3, a cell line maintained in semisolid agar medium, no metaphase spreads were obtained.

Interphase Nuclei. In contrast to metaphase spreads, interphase nuclei from Germa 1 and 2 evaluated on the same slides revealed a small percentage of nuclei (≤5%) with only one hybridization signal. This result might indicate either a small fraction of interphase cells with monosomy of chromosome 1 or incomplete probe penetration. Such penetration problems may lead to an underestimation of hybridization sites present in a given nucleus, and it may be difficult to distinguish such cases from the actual loss of chromosomal targets (7). In previous experiments normal diploid interphase cells hybridized on slides with pUC1.77 showed in addition to the predominant fraction of nuclei with two signals (≃68%) a minor fraction with only one signal (≃20%) or even no signal at all (≃9%) (7). To improve probe penetration nuclei were isolated from normal lymphocytes and hybridized in suspension. Under these conditions 98% of nuclei showed two spots. Nuclei with three spots were not observed, and only 2% yielded one spot. When isolated nuclei from the tumor cell lines Germa 1, 2, and 3 were hybridized in suspension 1, spot nuclei were no longer observed in the evaluated samples. These results do not indicate the presence of a subpopulation of cells with monosomy of chromosome 1 (at least of the 1q12 subregion) in these tumor cell lines. We conclude that hybridization of isolated nuclei in suspension was more efficient than hybridization of interphase cells on slides and might therefore present a more reliable method to screen for subpopulations of interphase nuclei with monosomies or nullosomies.

However, extra copies of the 1q12 region could be detected with high reliability in interphase cells from all three cell lines independent from the type of preparation. In Germa 1 the mean spot numbers obtained for metaphase spreads (3.00), for corresponding interphase nuclei on slides (2.92), and for interphase nuclei hybridized in suspension (3.04) did not significantly differ ($p < 0.01$) as tested by the Mann-Whitney U test (28). In Germa 2 no significant difference in mean spot numbers was observed for metaphase spreads (2.98) and corresponding interphase nuclei (2.90), whereas mean spot numbers obtained for interphase nuclei in suspension (3.31) was significantly higher ($p < 0.01$). We interpret this result to reflect the increased hybridization efficiency of isolated nuclei in suspension, resulting in the detection of a fraction of Germa 2 nuclei with six signals (Table I). Although in Germa 1 and 2 the fraction of three spot

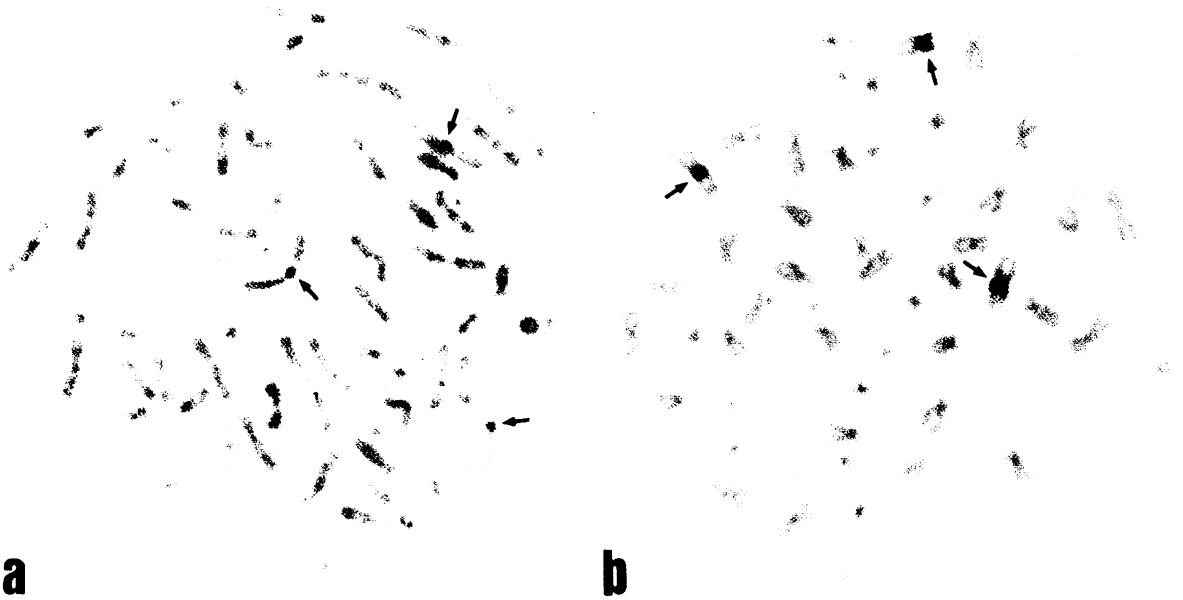


FIG. 1. Metaphase spreads from liquid cell cultures of Germa 1 (*a*) and Germa 2 (*b*) after *in situ* hybridization with biotinylated probe pUC1.77 (peroxidase detection). *Arrows*, specific labeling at 1q12 region of three chromosomes in each metaphase. One labeled chromosome in *panel b* shows a deletion of the major part of 1q (Giemsa: *a*, ×2100; *b*, ×2700).

TABLE 2. EVALUATION OF LABELED CHROMOSOMES IN 53 METAPHASE SPREADS (100%) FROM CELL LINE GERMA 2 AFTER *IN SITU* HYBRIDIZATION WITH BIOTINYLATED PROBE pUC1.77 (PEROXIDASE DETECTION)

| Labeled chromosomes | % |
|---|---|
| 2 chromosomes 1 | 9.4 |
| 1 chromosome 1 + 1 marker chromosome | 11.0 |
| 2 chromosomes 1 + 1 marker chromsome | 67.0 |
| 3 chromosomes 1 + 1 marker chromosome | 1.9 |
| 2 chromosomes 1 + 2 marker chromosomes | 1.9 |
| 4 chromosomes 1 + 2 marker chromosomes | 7.8 |

In each spread the number of apparently normal chromosomes 1 and chromosome 1-derived marker chromosomes was determined.

nuclei was predominant, in Germa 3 only a minor fraction of nuclei showed three signals (37%, mean spot number 2.38). Nevertheless Germa 3 in accordance with Germa 1 and Germa 2 showed a highly significant increase in mean spot numbers ($p < 0.001$) compared with the numbers obtained for normal diploid cells; see Table 1 for hybridization of isolated lymphocyte nuclei in suspension and Ref. 7 for experiments with diploid human fibroblasts and lymphocytes hybridized on slides. In conclusion our data confirm that interphase cytogenetics with probe pUC1.77 can be reliably used for the detection of hyperploidy of the 1q12 region, even if the respective interphase cells constitute only a minor fraction of the cell population.

## *IN SITU* DELINEATION OF 1Q12 IN PARAFFIN EMBEDDED TISSUE SECTIONS

Ideally, one would like to investigate chromosome aberrations in fresh primary tumor tissues or paraffin sections thereof. To develop a reliable and routinely applicable method we have used paraffin embedded tissue sections obtained from apparently normal human intestine and testis tissue as well as from xenografted male germ cell tumors that correspond to the examined cell lines. Tissue fixation, thickness of tissue sections, and permeabilization procedures performed before *in situ* hybridization are essential steps with regard to probe penetration and hybridization efficiency. In the following we describe experiments that were intended to optimize these parameters. If not stated otherwise a standard protocol, described in "Material and Methods," was followed except for the parameters in question.

Four fixation protocols were tested. Tissue fixed for 3–7 hours with Bouin's solution or with a mixture of 2% Formalin and 0.2% glutaraldehyde yielded weak hybridization signals in about 10% of the nuclei or no signals at all (90%). In paraffin sections fixed with 2% paraformaldehyde or 4% Formalin for the same period strong signals were observed in nearly all nuclei (>90%). Notably, hybridization efficiencies decreased with extended fixation times. Tissues routinely fixed overnight did not yield clearly detectable signals. Based on these results 4–5 hours fixation time with 2% paraformaldehyde or 4% Formalin were routinely employed in the following experiments.

The attachment of sections on microscope slides with aminoalkylsilane, as described by Rentrop *et al.* (38) for frozen tissue sections, provided an ideal adhesive support for paraffin sections. Other attachment procedures, such as poly-L-lysin, Elmer's Glue-All, or albumin were less convenient and led to the occasional loss of sections during the hybridization procedure.

Pretreatment steps were performed with proteinase K digestion (5–200 μg/ml) and HCl treatment (0.2 N HCl) of tissue sections to improve probe penetration. Although proteinase K digestion at the lowest concentration range resulted in insufficient hybridization efficiency, the morphology of tissue sections was drastically impaired at the

highest concentrations. In this context the thickness of sections was also important. Best results were obtained with 6-$\mu$m paraffin sections after pretreatment with 0.2 N HCl and digestion of protein with 50 $\mu$g proteinase K/ml. Sections 4 $\mu$m thick showed very sufficient hybridization (strong signals in >95% of nuclei) but a less well-preserved morphology, whereas 8-$\mu$m sections had reasonably good morphology with a considerably decreased percentage of hybridized nuclei (about 60%).

Detection of hybridized probes was performed either with a peroxidase reaction or indirect immunofluorescence using a fluorescein isothiocyanate-conjugated second antibody. Both detection procedures showed similar sensitivity with probe pUC1.77 (Fig. 2*a*, *b*). Experiments for the quantitative evaluation of serial section were performed by peroxidase detection, where counterstaining with hematoxylin allows better visualization of the morphology.

To assure that the normal diploid state of chromosome 1 can be demonstrated by *in situ* hybridization with probe pUC1.77, the above protocol was applied to histologically normal sections of human intestinal and testis tissues. Nuclei from normal intestinal tissue had an average diameter of 9 $\mu$m (range 5–11 $\mu$m) after *in situ* hybridization. Accordingly three 6-$\mu$m serial sections were simultaneously processed on one slide to assure the evaluation of whole nuclei. After *in situ* hybridization corresponding areas in the three sections were photographed, and only nuclei, localized entirely within the three sections, were evaluated. Most nuclei (86%) showed two hybridization signals, indicating two chromosomes 1 in each nucleus. Still the fraction of 1-spot nuclei (13%) was significantly higher ($p < 0.001$) compared with lymphocyte nuclei hybridized in suspension (2%). In addition to probe penetration problems, as described above for *in vitro* cultivated cells, 1-spot nuclei in sections from normal diploid tissues may result either from loss of target regions during tissue sectioning or indicate closely associated 1q12 regions (see also below). In sections of two histologically normal seminiferous tubules all cells except later stages of spermatids and spermatozoa were evaluated. The latter cells did not show sufficient hybridization in the present experiments (<10% of labeled nuclei). Normal spermatogonia typically showed two hybridization signals, which often were clearly separated (Fig. 3*a*). In primary spermatocytes the signals were also separated in some cases, whereas in others one particularly large hybridization spot was obtained, indicating that the 1q12 sites were already paired in these cases (Fig. 3*c*, *d*). In early spermatids only one smaller signal was seen. Table 1 summarizes the results for the two completely evaluated tubuli sections. Notably, no cells with three hybridization signals were observed. In one case a spermatogonium with four signals was detected.

Tumor xenografts from which the above three tumor cell lines were derived were processed in the same way. The diameter of tumor nuclei (range 8–15 $\mu$m, mean value 12 $\mu$m) was considerably larger compared with nuclei from normal intestinal epithelium (see above) or normal spermatogonia (range 4–10 $\mu$m, mean value 7 $\mu$m). Although few particularly large nuclei were excluded from further evaluation by our protocol based on three consecutive sections with 6 $\mu$m each, sufficient numbers of apparently complete nuclei were still obtained. Most nuclei (65–75%) in these tumor sections presented three clearly separated hybridization signals (Table 1, Fig. 4). Results from tumor tissue sections were in reasonable agreement with the results described above for the respective tumor cell lines Germa 1 and 2 but not for Germa 3 (Table 1). The reason for the difference in the observed peak fractions (three spots *versus* two spots; $p < 0.01$) between tissue sections and the respective cell line Germa 3 is unclear. Nevertheless in contrast to nuclei evaluated from normal tissue sections, tissue sections from all three tumors showed a highly significant fraction of nuclei with three spots ($p < 0.001$). In conclusion our data demonstrate that interphase cytogenetics applied to tissue sections is a valid method to demonstrate subpopulations of cells with numerical chromosome aberrations.

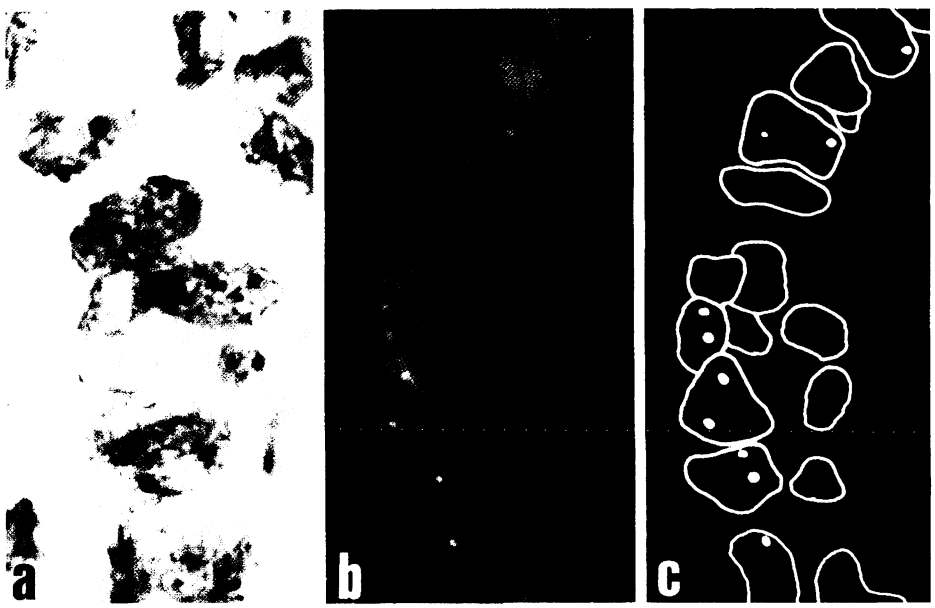


FIG. 2. Paraffin section (6 $\mu$m) of Formalin fixed normal intestinal mucosa after *in situ* hybridization with biotinylated probe pUC1.77 and peroxidase detection (*a*) or fluorescein isothiocyanate (*b*). Nuclei and hybridization spots shown in *panel b* are schematically represented in *panel c*. In some nuclei both hybridization signals are in focus. In others one or even both signals were out of focus or not contained within the same section plane. (*a*: hematoxylin, ×2100; *b*: propidiumiodide, ×2000).

## Present Stage and Future Prospects of Interphase Cytogenetics

Interphase cytogenetics has recently become a new and potent tool to analyze numerical and structural chromosome aberrations directly in tumor cell nuclei (7, 17). In this paper we describe in detail reliable procedures by which this new approach is routinely applied to isolated nuclei in suspension and paraffin embedded tissue sections. These procedures can be used in cases where *in vitro* cultivation is not feasible and should open new avenues for pathologists to investigate single tumor cells for specific chromosome aberrations. Compared with cells fixed on slides nuclei in suspension have yielded a very high fraction (close to 100%) of apparently complete hybridization. This methodological improvement seems important in cases where a low percentage of cells with a specific chromosome loss should be detected. *In situ* hybridization of isolated nuclei in suspension may be particularly useful for studies of cells from body fluids such as blood, seminal fluid, or pathologic fluids obtained by puncture or smears such as Pap smears from the female genital tract and other regions for specific chromosome aberrations without necessity of prior cultivation *in vitro*.

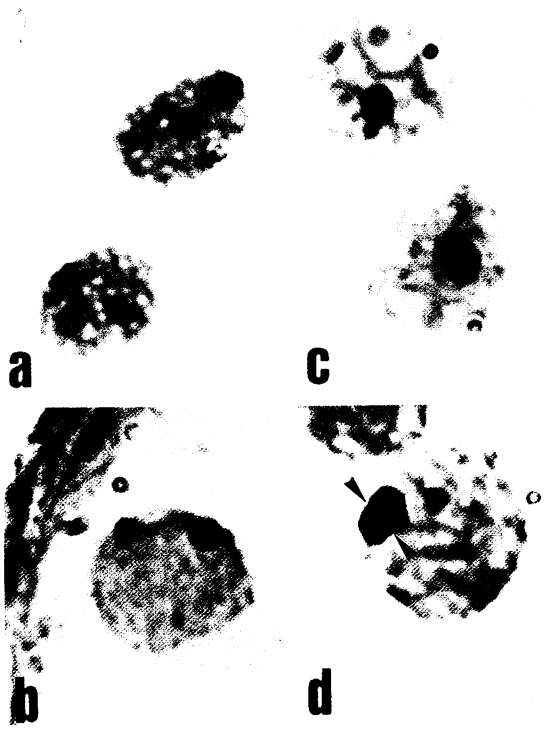


FIG. 3. Spermatogonia (*a*, *b*) and primary spermatocytes (*c*, *d*) after *in situ* hybridization with probe pUC1.77 (peroxidase detection). Due to an excessive diaminobenzidine-reaction hybridization signals are particularly large in this case. In spermatogonia two hybridization signals are clearly separated. In contrast primary spermatocytes show only one apparent signal or two closely adjacent signals (see *arrows* in *d*) (hematoxylin, ×1800).

Interphase cytogenetics of tissue sections derived from biopsies or surgical material has the advantage that the histological context of different cell types is preserved. We have applied this approach to atypical germ cells of testis biopsies. These cells are considered as carcinoma *in situ* of the testis and can often be found in seminiferous tubules from patients with germ cell tumors and also occasionally from patients with fertility problems. In these cells we have demonstrated the presence of three chromosomes 1 compared with two chromosomes 1 in normal spermatogonia (H. Walt *et al.*, unpublished data). Interphase cytogenetics may thus provide new diagnostic criteria to discriminate between normal and abnormal cells even at early stages of tumorigenesis.

Two present limitations of this approach concern first the lack of DNA probes suitable for specific staining of chromosomal targets of diagnostic importance and sec-

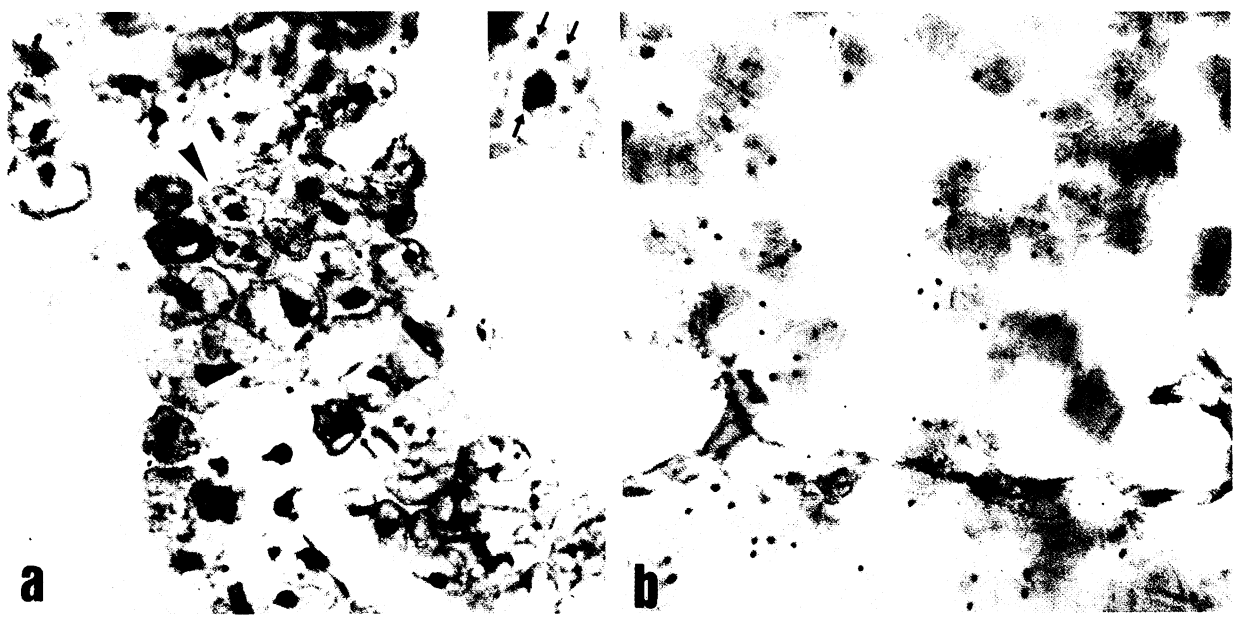


FIG. 4. Paraffin sections (6 μm) of Formalin fixed xenografts from tumor 1 (*a*), tumor 3 (*b*) after *in situ* hybridization with biotinylated probe pUC1.77 (peroxidase detection). Both tumors are phenotypically embryonal carcinomas and exhibit numerous polymorphic nuclei with three hybridization signals; see also *panel a inset* (hematoxylin: *a*, ×1000; *inset*, ×1700; *b* ×1200).

ond the lack of procedures for the differential staining of many such targets within a given nucleus. Both limitations should be overcome soon as briefly discussed below.

DNA probes useful to label chromosome specific target DNAs in the constitutive heterochromatin are already available for numerous human chromosomes such as 1, 4, 5, 6, 7, 9, 11, 13, 14, 15, 16, 17, 18, 19, 21, 22, X and Y (Table 3). Recently additional probes have been published that detect specific repetitive target sequences on chromosome arms (2, 31), and many more such probes may be expected in the future. In addition the number of useful probes will very much increase with future improvements of nonradioactive *in situ* hybridization techniques. Only a few kilobase target sequences can now be detected in mitotic chromosomes and interphase nuclei (27), and pools of single copy sequences can be used to delineate specific chromosomal subregions such as 21q22.3 required to elicit the Morbus Down phenotype (27). Moreover it has become possible to delineate complete individual chromosomes at any stage of the cell cycle (6, 26, 36). This new approach makes use of phage DNA libraries established from sorted human chromosomes. Human inserts are isolated, chemically modified, and used as highly complex probes for *in situ* hybridization. Hybridization of nonchromosome specific sequences in these probes can be sufficiently suppressed by preannealing steps using an appropriate excess of nonlabeled human genomic DNA. By this technique normal and aberrant chromosome domains can be directly visualized in tumor nuclei (6). *In situ* suppression hybridization has also successfully been applied to cosmid clones with large human inserts containing both chromosome specific and highly repetitive sequences (23).

A second very important development concerns techniques for the simultaneous use of various DNA probes chemically modified in different ways such as biotin (24), mercury (18), acetylaminofluoren (22), sulfur (11), BrdU (34), and DNP (39). Triple hybridization protocols have been successfully applied to detect three differently colored chromosome targets simultaneously (32). The possibilities to chemically modify DNA probes for nonradioactive *in situ* hybridization are steadily increasing, and we expect that the simultaneous delineation of many more targets can soon be realized. The full potential of the methodology presented in this paper will be realized in the context of these new and rapid developments.

TABLE 3. CHROMOSOME SPECIFIC REPETITIVE DNA PROBES

| Chromosome localization | Name | Repetitive family | Ref. |
|---|---|---|---|
| 1q12 | pUC1.77 | Sat III | 3 |
| 1c | pSD1.1 | alphoid | 44 |
| 1p36 | p1-79 | unknown | 2 |
| 4c/9c | pG-Xball | alphoid | 19 |
| 5c/19c | pG-A16 | alphoid | 19 |
| 6c | p308 | alphoid | 20 |
| 7c | p7dl | alphoid | 45 |
| 7c | p7t1 | alphoid | 45 |
| 8c | λ-285 | alphoid | 10 |
| 9c | pHuR98 | Sat III | 30 |
| 11c | pLC11A | alphoid | 43 |
| 13c/21c | L1.26 | alphoid | 9 |
| 14c/22c | αXT (680) | alphoid | 21 |
| 15c | D15Z1 | Sat III | 15 |
| 16c | pHuR195 | Sat II | 30 |
| 17c | P17H8 | alphoid | 44 |
| 18c | L1.84 | alphoid | 9 |
| 22c | p22/1:2.1 | alphoid | 29 |
| Xc | pBamX5 | alphoid | 47 |
| Xc | pXBR | alphoid | 48 |
| Yp | Y-190 | unknown | 31 |
| Yq | pHY2.1 | Sat III | 4 |

## METHODS

### PATIENTS AND ANIMALS

Tumors from three patients were subcutaneously grown in NMRI nude mice as described earlier (33, 42). Tumors 1 and 2, two teratocarcinoma, were in their 17th and 34th xenograft generation, whereas tumor 3, an embryonal carcinoma, was in the 42nd generation at time of preparation.

### CELL CULTURES

Cells from xenografts of tumors 1 and 2 grew both as monolayers in liquid cultures and in solid agar medium, whereas cells from xenografts of tumor 3 were grown in semisolid agar medium (42, M. C. Hofmann *et al.*, unpublished data). These cell lines were termed GERMA 1–3. Culture conditions briefly follow: 1) Semisolid agar medium was prepared as described by Hamburger and Salmon (14). Tumors were collected from nude mice at passages 9 (tumor 1), 2 (tumor 2), and 38 (tumor 3) and were dispersed mechanically into single cell suspension. 2) Liquid cultures were inoculated with cells growing in the above agar medium for 59 passages (Germa 1 and 2), transferred into 25-$cm^2$ tissue culture flasks (Falcon), and cultured in enriched CMRL 1066 medium. Confluent growth was observed after 2 weeks incubation at 37°C and 5% $CO_2$.

### METAPHASE PREPARATION FROM LIQUID CELL CULTURES

After an exposure to colcemid (0.1–0.2 μg/ml) for 4 hours, cells from passages 15–20 were detached with 0.05% trypsin/0.02% EDTA, harvested by centrifugation (10 min, 1000 rpm), and treated with hypotonic solution (0.035 M KCl) for 20 minutes at 37°C. Cells were fixed three times with methanol/acetic acid (3:1), dropped onto slides, stored for at least 1 hour in 99% ethanol at 20°C, and then dipped into ice-water. After air drying these preparations were stored in 70% ethanol at 4°C before *in situ* hybridization.

### PREPARATION OF NUCLEI IN SUSPENSION FROM ISOLATED LYMPHOCYTES, AGAR CELL CULTURES AND LIQUID CELL CULTURES

Nonstimulated lymphocytes were isolated from healthy female and male humans using Lymphoprep (Nyegaard, Oslo, Norway) according to the producers instructions. Colonies from Germa 3 were collected in CMRL 1066 medium after 25 passages in semisolid agar medium. Cells from liquid cell cultures (passage 17 for Germa 1 and passage 18 for Germa 2) were harvested as described for metaphase preparations. All cell suspensions were centrifuged, and the cell pellet was washed in Hanks' balanced salt solution (Serva, Heidelberg, FRG). For isolation of nuclei the method described by Trask *et al.* (40) was simplified as follows. The pellet was resuspended in 1 ml hexyleneglycol isolation buffer (25 mM Tris-Cl, 750 mM 1.6-hexanediol, 5 mM $MgCl_2$, pH 3.2) to disrupt the cell membrane and isolate the nuclei. Nuclei were washed in the isolation buffer, and the suspension of nuclei was stored at 4°C in 1 ml

fresh isolation buffer. All centrifugation steps were performed at 1000 rpm for 10 minutes.

### Tissue Preparation

Tumor transplants (5 mm) were fixed for 4–5 hours in freshly prepared 2% paraformaldehyde or in 4% Formalin in phosphate buffer. Normal intestinal tissue from another patient was fixed in 4% Formalin in phosphate buffer. Specimens were then dehydrated over a graded series of ethanols and embedded in Paraplast. Sections of 6 μm were attached on microscope slides treated with aminoalkylsilane, as detailed by Rentrop *et al.* (38). To allow analysis of entire tumor nuclei small series of two to three subsequent sections per slide were processed for *in situ* hybridization (see below).

### Pretreatment of Paraffin Embedded Tissue Sections

Microscope slides with attached sections were stored at −70°C in a plastic bag containing Silicagel. To facilitate probe penetration, sections were subsequently thawed and frozen five times at 30-minute intervals, then incubated at 65°C overnight. Paraffin was removed by xylene and ethanol (99%) for 5 minutes each. Air dried slides were then treated with 0.2 N HCl for 20 minutes at room temperature and rinsed with phosphate-buffered saline (PBS). Proteinase K (Boehringer, Mannheim, FRG) digestion was performed for 10 minutes at 37°C at a concentration of 50 μg proteinase K/ml PBS. The reaction was stopped by rinsing with PBS containing 5 mM EDTA for 10 seconds. Samples were further rinsed in PBS, dehydrated over a graded series of ethanols (70–90–99%) followed by acetone (2 minutes each), and finally air dried.

### Labeling Procedure for DNA Probe pUC1.77

Probe pUC1.77 was a generous gift from Howard Cooke. It represents a 1.77-kb EcoRI fragment from human satellite DNA fraction III (3) and was cloned in the plasmid vector pUC9. The insert represents a tandem repeated sequence derived from the region 1q12. The probe was nicktranslated with biotin-11-dUTP using a commercial kit from Bethesda Research Laboratories (Rockville, Maryland).

### *In Situ* Hybridization Conditions

Hybridization of Cells on Slides. The best hybridization results were obtained with a mixture containing 60% formamide, 2 × SSC (SSC: 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0), 5% dextrane sulfate, 100 μg/ml herring sperm DNA, and 2 μg/ml labeled DNA probe; 10 μl of the mixture for each centimeter squared of the specimen were added, and the slide was sealed with a coverslip. Specimens and probe were denatured simultaneously in a moist chamber at 76°C for 10 minutes. Hybridization occured at 40°C overnight under the same conditions. Slides were washed at room temperature twice for 15 minutes each in 50% formamide/1 × SSC and then at 37°C for 30 minutes in 0.1 × SSC.

Hybridization of Nuclei in Suspension. Before hybridization, the isolation buffer was removed by centrifugation, and the nuclei were washed in 1 ml 2 × SSC. The pellet was resuspended in 1 ml 2 × SSC/formamide, pH 7.0 (1:1, v:v), and transferred to a Falcon tube (no. 2054). The nuclei were again pelleted by centrifugation and resuspended in 0.5 ml hybridization mixture (see above) containing 1 μg/ml biotin-labeled DNA probe. The nuclei and probe were denatured simultaneously at 72°C for 10 minutes and hybridized overnight at 40°C. Unbound probe was removed by washing nuclei in 2 ml 2 × SSC at 37°C for 10 minutes and in 2 ml 0.1 × SSC at 37°C for 10 minutes. Thereafter the pellet of nuclei was resuspended in 1 ml PBS.

### Detection of Hybridized DNA Probes

Cells on Slides. Biotinylated DNA probes were visualized by a peroxidase reaction (DETEK I-hrp Kit, ENZO Ortho Diagnostik, Heidelberg, FRG) or with a double antibody fluorescence system (DETEK I-f, ENZO). Peroxidase preparations were counterstained with Mayers' hematoxylin (Sigma, St. Louis, Missouri); fluorescence preparations were counterstained with propidium iodide and mounted in DePeX (Serva), containing 2% β-mercaptoethanol.

Nuclei in Suspension. Biotinylated DNA probe was visualized by a peroxidase reaction (DETEK I-hrp Kit ENZO). For microscopy the nuclei suspension was dropped on a slide, stained with Giemsa, and mounted in Kaysers glycerol gelatin (Merck, Darmstadt, FRG).

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