Isolation of DNA probes specific for rat chromosomal regions 19p, 19q and 4q and their application for the analysis of diethylstilbestrol-induced aneuploidy in binucleated rat fibroblasts

Joyce M.de Stoppelaar, Petra Faessen, Edwin Zwart, Leo Hozeman, Hennie Hodemaekers, Georges R.Mohn and Barbara Hoebee

Laboratory of Health Effects Research, National Institute of Public Health and Environment, PO Box 1, 3720 BA Bilthoven, The Netherlands

DNA probes specific for rat chromosomes 19p, 19q and 4q were isolated, characterized and used for the detection and analysis of diethylstilbestrol (DES)-induced aneuploidy. By denaturing and partially reassociating total genomic DNA a new rat repetitive DNA family was isolated, which was located on chromosome 19p21. Sequencing of a number of subclones from cos76-1 and other clones of this so-called 76-family revealed that the repeat units are interrupted with large areas of other (unique) DNA. Consequently, after fluorescence in situ hybridization (FISH) the signals in interphase nuclei are large and spread out. The other two probes, cos25 (chromosome 4q) and cos42-47 (chromosome 19q), were isolated by screening cosmid libraries with probes isolated previously in our laboratory. The repeat unit of cos25 is a 2174 bp long EcoRI unit that contains three Sau3A sites and is tandemly organized. Sequencing of subclones of cos42-47 revealed that this probe was in fact the SS RNA gene, located on 19q12. In order to determine if these probes were suitable probes for aneuploidy detection, two series of dual colour FISH with the combinations cos25/cos76-1 (4q/19p) and cos42-47/cos76-1 (19q/19p) were carried out on slides from an in vitro micronucleus assay with DES. With all three probes used, an increase in binucleated cells with non-disjunction or chromosome loss was observed in the DES-treated cultures. Scoring of additional micronucleated cells on slides hybridized with the cos25/cos76-1 (4q/19p) probes revealed that the hybridization signal of probe cos25 (4q) was over-represented in the micronuclei of the control cultures. The simultaneous use of the 19q and 19p probes is a particularly valuable tool for the detection of aneuploidy, since it allows distinction between aneugenic and clastogenic events in binucleated cells. Results of this analysis showed that apart from aneuploidy, DES also induced structural chromosome aberrations, although to a lesser extent.

Introduction

Numerical chromosome aberrations, or aneuploidy, is a common class of chromosome aberrations encountered in humans. It is the leading cause of pregnancy loss, congenital abnormalities and mental and physical retardation (Hassold and Jacobs, 1984; Griffin, 1996; Hassold et al., 1996). In addition, certain aneuploidies are consistently associated with some types of human cancer and may play a significant role in the formation and progression of tumours (Oshimura and Barret, 1986; Holliday, 1989). Experimental evidence suggests that certain chemicals, such as colchicine, carbendazim and diethylstilbestrol (DES), are able to induce aneuploidy by interacting with the spindle apparatus or other cell organelles involved in chromosome segregation (Liang and Brinkley, 1985). Considering the adverse effects of the occurrence of aneuploidy, the need for the development of well-validated assays for aneuploidy detection is well recognized (Adler and Parry, 1993; Parry J.M., 1996; Marzin, 1997).

Over the past few years the in vitro cytokinesis-blocked micronucleus (MN) assay in human lymphocytes (first described by Fenech and Morley, 1985), in combination with fluorescence in situ hybridization (FISH), has become a widely used method for detecting aneuploidy in dividing cells. Incubation with cytochalasin B produces binucleated cells, in which aneuploidy can be scored after FISH with, for example, chromosome-specific probes (Boei and Natarajan, 1995; Doherty et al., 1996; Kirsch-Volders et al., 1997; Perrallés and Natarajan, 1997). A major advantage of this technique is that both non-disjunction and chromosome loss, the two basic mechanisms by which aneuploidy arises, can be detected in the same cells.

Ideally, chromosome-specific probes used for aneuploidy detection should hybridize in the centromeric region of the chromosome and give compact signals in interphase nuclei after FISH. Tandemly repeated DNA families have been shown to be suitable candidates for such probes, since they are organized in tandem arrays and remain in a condensed state throughout (most of) the cell cycle, resulting in bright and distinct signals in interphase nuclei (Cremer, 1986; Devilee et al., 1988). For (almost) all human chromosomes such chromosome-specific probes have been isolated, consisting of α-satellite DNA (Willard and Waye, 1987). For laboratory animals the situation is less favourable and only a limited number of such DNA probes are available for, for example, mouse and hamster. For the rat, such probes were not available when we started this work and we therefore isolated several rat chromosome-specific probes in our laboratory (Essers et al., 1995; Hoebee and de Stoppelaar, 1996). This paper describes the isolation and characterization of three chromosome-specific probes, namely for chromosomal regions 19p, 19q and 4q. In addition, the isolated probes were tested for their usefulness in the detection of aneuploidy in a cytokinesis-blocked MN assay, using rat fibroblasts treated in vitro with DES.

Materials and methods

Isolation of repetitive rat DNA

Rat double-stranded (ds)DNA was sonicated into fragments of ~500 bp, denatured in 0.1× SSC for 5 min at 100°C, followed by renaturation overnight at 1 h at 65°C. DNA was desalted using a Sephadex G-100 spin column equilibrated with 0.1× SSC. dsDNA was separated from single-stranded (ss)DNA on a hydroxyapatite column. Both ssDNA and dsDNA fractions were concentrated with 2-butanol, desalted as described above and precipitated with ethanol. The ssDNA fraction was dissolved in 0.1× SSC and again

1To whom correspondence should be addressed. Tel: +31 30 274 3632; Fax: +31 30 274 4446; Email: b.hoebee@rivm.nl

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Table I. Summary of the isolation of discussed phages and cosmids and their localization after FISH

<table>
<thead>
<tr>
<th>Probe used for library screening</th>
<th>Isolated clones</th>
<th>Localization</th>
<th>FISH signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product of phage 9.1&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Cosmid 25</td>
<td>4q</td>
<td>Bright and distinct signal</td>
</tr>
<tr>
<td>PCR product of chromosome 20&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Cosmid 42</td>
<td>Xq</td>
<td>Distinct signals on multiple sites along the X chromosome</td>
</tr>
<tr>
<td>Cosmid 42</td>
<td>Cosmid 42-47</td>
<td>19q</td>
<td>Bright and distinct signal</td>
</tr>
<tr>
<td>Reassociation clone rr76</td>
<td>Phage 76-11</td>
<td>19p</td>
<td>Large but spread out signal in interphase</td>
</tr>
<tr>
<td>Reassociation clone rr76</td>
<td>Phage 76-14</td>
<td>19p</td>
<td>Large but spread out signal in interphase</td>
</tr>
<tr>
<td>Reassociation clone rr76</td>
<td>Cosmid 76-1</td>
<td>19p</td>
<td>Large but spread out signal in interphase</td>
</tr>
</tbody>
</table>

<sup>6</sup>PCR product was obtained using primers specific for the 60-56 family described by Essers et al. (1995).

Table II. Summary information on subclones used for sequencing

<table>
<thead>
<tr>
<th>Phage/cosmid clones</th>
<th>Digested with</th>
<th>Subclone</th>
<th>Insert (kb)</th>
<th>Sequence status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phage 76F11</td>
<td>BamHI</td>
<td>76F11B3</td>
<td>0.71</td>
<td>Both strands</td>
</tr>
<tr>
<td>Phage 76F14</td>
<td>BamHI</td>
<td>76F14B7</td>
<td>0.67</td>
<td>Both strands</td>
</tr>
<tr>
<td></td>
<td>HindIII</td>
<td>76F14H49</td>
<td>1.53</td>
<td>Both strands</td>
</tr>
<tr>
<td></td>
<td>Sau3A</td>
<td>76F14H63</td>
<td>1.53</td>
<td>Both strands</td>
</tr>
<tr>
<td>Cosmid 76-1</td>
<td>HindIII and</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BamHI</td>
<td>76-1B3b</td>
<td>1.95</td>
<td>Both strands</td>
</tr>
<tr>
<td>Cosmid 25</td>
<td>EcoRI and</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BamHI</td>
<td>76-1EB1-2</td>
<td>2.0</td>
<td>Both strands</td>
</tr>
<tr>
<td>Cosmid 42-47</td>
<td>SmaI</td>
<td>42-47Sma1</td>
<td>0.84</td>
<td>One strand</td>
</tr>
<tr>
<td></td>
<td>Sau3A</td>
<td>42-47Sau1</td>
<td>1.7</td>
<td>Partial</td>
</tr>
<tr>
<td></td>
<td>Sau3A</td>
<td>42-47Sau3</td>
<td>1.7</td>
<td>Partial</td>
</tr>
</tbody>
</table>

from randomly picked colonies were used as probes in FISH. A list of the studied subclones with their inserts is shown in Table II.

Southern blot and sequence analysis

High molecular weight DNA isolated from rat liver was digested with EcoRI, Sau3A and HindIII, separated on a 1% agarose gel and transferred to Genescreen filters. The insert of cos25 was used as probe, which was isolated by digestion of cos25 with NcoI. After gel electrophoresis the insert was excised followed by Spin X centrifugation. The probe was labelled with <sup>32</sup>P and the filters were hybridized as described previously (Essers et al., 1995).

Sequence reactions were performed on a DNA Labstation 625 (Vistra, Amersham) using (i) a Labstation Thermo Sequenase labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham RPN2438) and 21 M13 dye primers (Applied Biosystems) or (ii) a Labstation Thermo Sequenase fluorescent dye-terminator cycle sequencing kit (Amersham RPN2435) in combination with a magnetic precipitation and purification kit (Amersham RPN2339) and oligonucleotides (Eurogentec). The samples were loaded on a 4% polyacrylamide gel and run on a DNA sequencer 373A (Applied Biosystems).

To investigate homology of the obtained sequences with other sequences we used the BLASTN program (Altschul et al., 1990) and all sequences submitted to the GenBank, EMBL, DDBJ and PDB databases.

Metaphase and MN slides

The metaphase slides used for testing of the isolated probes were prepared from untreated primary fibroblasts following standard procedures. MN slides from a previously described in vitro cytokinesis-blocked MN assay with DES (10 and 13.5 μg/ml) in rat fibroblasts were used for aneuploidy detection with the isolated probes (see de Stoppelaar et al., 1997). All slides were stored at −20°C until use.

Probe labelling and FISH

DNA of reassociation clones, whole cosmids, phages or subclones were used as probes in the FISH experiments and were all labelled with biotin-dUTP (Boehringer Mannheim) by nick translation. For the detection of aneuploidy the following probes were used: cos76-1 labelled with fluorescein-dUTP (Boehringer Mannheim) and probes 25SS and cos42-47 labelled with biotin-dUTP (Boehringer Mannheim). All probes were dissolved in hybridization mixture (50% formamide, 2× SSC, 50 μM phosphate, 10% dextran sulphate, pH 7) and denatured at 80°C.

Metaphase spreads were directly used whereas MN preparations were heated on a hotplate (56°C) for a few hours. FISH was performed as described previously (de Stoppelaar et al., 1994), except for the prehybridization step, which was omitted. DNA on the slides was denatured at 70°C in 70% formamide, 2× SSC, 50 μM phosphate buffer for 3 min and dehydrated in ethanol series. Approximately 100 ng probe per drop of cells was hybridized overnight at 37°C in a humidified chamber. Following hybridization, the slides were washed three times in 2× SSC at 65°C and three times in 0.1× SSC at 45°C. The biotinylated probe was detected by applying alternate layers of avidin–c–y3 (Jackson ImmunoResearch) and biotinylated anti-avidin (Vector Laboratories). After dehydration, the slides were mounted in antifade medium containing DAPI as counterstain.

Scoring of the slides

Scoring of the slides was performed using a Zeiss Axiophcope fluorescence microscope equipped with the appropriate filters. Binnucleated cells were scored for the presence of MN and the distribution of the hybridization signals of the probes between the two daughter nuclei and MN (if present) was determined. The criteria for binnucleated cells and MN described by Fenech (1993) were used in the analysis. Furthermore, the hybridization signals were analysed according to the following criteria: (i) scoring was only performed in those areas of the slide where the hybridization signals were clearly visible;

denatured for 5 min, followed by renaturation over 3 h at 65°C. Separation of ssDNA and dsDNA was carried out as described above.

The dsDNA fractions were treated with Klenow polymerase and blunt-end ligated into a pUC18/SmaI vector (Pharmacia). The ligation mixture was transformed into Escherichia coli strain EC 490. After transformation at least 500 colonies were found on all plates. Colonies were transferred onto nitrocellulose filters and screened by hybridization following standard procedures, using a combination of the following 3<sup>2</sup>P-labelled probes: (i) PCR products with primers which are specific for the rat satellite I family (previously described by de Stoppelaar et al., 1997) on total rat DNA; (ii) a probe isolated in a previous reassociation experiment which hybridized with interspersed repetitive sequences along the chromosomes. DNA of negative colonies was isolated using standard procedures and directly used for FISH.

Screening of a phage or cosmid library

Two different genomic libraries were screened: (i) a rat genomic phase λ library containing a partial Sau3A digest of DNA isolated from an adult male Sprague–Dawley rat and cloned in vector EMBL-3 Sp6/T7 (Clontech); (ii) a rat genomic cosmid library containing partial Sau3A1 digests of DNA isolated from the brain of a male Wistar Kyoto rat and cloned in a SuperCos1 Vector (Stratagene). The libraries were screened according to the protocol of the manufacturer using a number of different probes, which are summarized in Table I.

Subcloning of phages and cosmids

Phages 76F11 and 76F14 and cosmids 25, 42-47 and 76-1 were subcloned into pUC18 (see Table II) following standard procedures. Briefly, the phages and cosmids were digested with the restriction enzymes mentioned in Table II and separated on a 1% agarose gel. Those bands that were more intense than expected (and probably containing more than one fragment of the same size) were excised. DNA was isolated using a Spin X column and directly ligated into pUC18 vector digested with the enzymes as listed in Table II, except for the Sau3A digests, which were cloned in the pUC18 BamHI site.

All ligation products were transformed into E.coli EC 490 and plasmids...
As we were interested in new DNA families, which are Y (near the telomere) and becomes chromosome Y specific of chromosomes 3 and 12 and on the long arm of chromosome repetitive DNA family that hybridizes strongly on the satellites family). However, one clone (60-56) contained a novel rat (the LINE family) or centromeric DNA (the rat satellite I obtained clones contained long interspersed repeated DNA or 60 min and cloning the dsDNA fraction, most of the 1995). After reassociating total rat (sonicated) ssDNA for 30 

Results and discussion
The centromeres of human chromosomes contain a repetitive DNA family, the α-satellite DNA family, which consists of 171 bp repeats organized in tandem arrays (Willard and Waye, 1987). The higher order α repeat organization found on the different chromosomes, together with the nucleotide sequence, is specific for that chromosome. Such chromosome-specific α-satellite probes are very suitable for aneuploidy detection and are readily (commercially) available for almost all human chromosomes. The centromeres of rat chromosomes contain the rat satellite I DNA, although this family is present only on ~80% of the chromosomes (de Stoppelaar et al., 1997). An attempt was made to isolate chromosome-specific probes of this DNA family, but this was unsuccessful, presumably due to the fact that the rat satellite I DNA may not be organized in the same way as the human α-satellite DNA. Therefore, our efforts focused on the isolation of other repetitive DNA families.

As these repetitive DNA families are more abundantly present in the genome than single copy genes, isolation of these families was achieved by reassociating ssDNA fragments. We have previously described the isolation of a rat repetitive DNA family (60-56 family) using this approach (Essers et al., 1995). After reassociating total rat (sonicated) ssDNA for 30 or 60 min and cloning the dsDNA fraction, most of the obtained clones contained long interspersed repeated DNA (the LINE family) or centromeric DNA (the rat satellite I family). However, one clone (60-56) contained a novel rat repetitive DNA family that hybridizes strongly on the satellites of chromosomes 3 and 12 and on the long arm of chromosome Y (near the telomere) and becomes chromosome Y specific after more stringent washings (Essers et al., 1995). This DNA family was used to isolate two of the chromosome-specific probes described in the present paper, namely probe 2555, hybridizing on chromosome 4q, and cos42-47, hybridizing on chromosome 19q. The third chromosome-specific probe, cos76-1, hybridizing on chromosome 19p, belongs to a new repetitive DNA family (the 76-family) which was isolated by performing a second reassociation of the ssDNA for another 3 h. The strategies followed to isolate and characterize the different probes are described below.

Isolation and characterization of a new rat repetitive DNA family (the 76-family)
A new rat repetitive DNA family was isolated by performing a reassociation experiment in two steps. In the first step DNA fragments of total genomic rat DNA (obtained by sonication) were denatured, followed by reassociation for 1 h, and the dsDNA and ssDNA fractions were separated. In a previously described experiment (Essers et al., 1995) it was determined that this dsDNA fraction contains repetitive DNA families that were already known (e.g. the rat satellite I and LINE families). As we were interested in new DNA families, which are probably less abundantly present in the genome, a second reassociation of the ssDNA fraction was carried out for another 3 h. The isolated dsDNA fractions of both the first (1 h) and the second (total 4 h) reassociation steps were cloned. After transfer of the obtained clones to filters, they were hybridized with a combination of the rat satellite I probes and a rat long interspersed repeat probe.

Eighty per cent of the clones isolated in the first reassociation step were positive and were not studied further. DNA from 36 negative clones was isolated and subsequently labelled for FISH on metaphase spreads. After FISH, most clones did not show a hybridization signal, whereas five clones showed an interspersed signal along all the chromosomes. As we do not exactly know which interspersed DNA family we have used as probe in the screening of the colonies, these five clones probably hybridize with a related repetitive family.

Of the clones isolated after the second reassociation step, 10–20% were found positive, illustrating that there was enrichment for other rat repetitive DNA families. DNA of 49 negative clones was isolated and, after FISH, seven of these clones showed hybridization signals. Two clones showed an interspersed signal on all chromosomes, which was also observed in the first reassociation step, and two other clones hybridized on the satellites of chromosomes 3 and 12 and near the telomere of chromosome Yq. This is the same hybridization pattern as we found for the 60-56 repeat family that we isolated in a previously described reassociation experiment (Essers et al., 1995). Two additional clones were chromosome Y specific and probably contained a subfamily of the 60-56 family (see also Essers et al., 1995). As the aim of the experiments was to isolate new repeat families, these six clones were not studied further.

The last positive clone after FISH analysis (clone rr76) hybridizes on the p arm of chromosome 19 (19p21, Figure 1A). Since a rat repetitive DNA family located at this chromosomal position had not been described before, this clone was studied in more detail.

Isolation and molecular characterization of the chromosome 19p probe (cos76-1)
Clone rr76 was sequenced and had an insert of only 177 bp (Figure 2). After FISH, the hybridization signal was not very strong (Figure 1A). In order to isolate clones with larger hybridization signals (being more useful for aneuploidy detection), both a rat genomic phage and a cosmid library were screened. Most of the isolated phages and cosmids hybridized on the p arm of chromosome 19 and showed a larger hybridization signal in interphase nuclei than the original rr76 clone (Figure 1B). In interphase nuclei the signal of these probes (and also from rr76) was often diffuse and spread out (Figure 1B), which may be due to interruption of the repeat unit by

<table>
<thead>
<tr>
<th>DES (µg/ml)</th>
<th>Total scored BC with n MN</th>
<th>MNBC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1518</td>
<td>1490</td>
</tr>
<tr>
<td>0</td>
<td>1575</td>
<td>1548</td>
</tr>
<tr>
<td>10</td>
<td>1522</td>
<td>1456</td>
</tr>
<tr>
<td>10</td>
<td>1575</td>
<td>1523</td>
</tr>
<tr>
<td>13.5</td>
<td>1466</td>
<td>1358</td>
</tr>
<tr>
<td>13.5</td>
<td>1521</td>
<td>1387</td>
</tr>
</tbody>
</table>

BC, binucleate cells; MN, micronuclei; MNBC, micronucleated binucleate cells.
Fig. 1. FISH with clone rr76 (A), cosmid 76-1 (B), cosmid 25 (C) or cosmid 42-47 (D) on rat metaphases. The probes were labelled with biotin-dUTP and detected with avidin–FITC; propidium iodide or DAPI was used as counterstain. Dual colour FISH with cosmid 76-1 (19p, labelled with FITC–dUTP) and plasmid 25S5 (4q, labelled with biotin–dUTP and detected with avidin–cy3) on binucleated cells, showing non-disjunction of chromosome 19 (E) or loss of chromosome 4 (F). DAPI was used as counterstain.

Fig. 2. Nucleotide sequence of the insert of reassociation clone rr76.

other (unique) sequences. This was supported by the sequence analysis of the subclones.

Two phages (76F11 and 76F14) and one cosmid (cos76-1) were subcloned using different restriction enzymes. Those subclones that showed a signal on chromosome 19 after FISH were sequenced, revealing a large sequence homology among the clones (schematically represented in Figure 3). The sequence of clone rr76 was present in subclones of both the isolated phages (subclones 76F14H49 and 76F11B3) and the cosmid (76-1HB-23). The observed sequence homology was >92%. Among the three subclones the observed sequence homology was >90% (Figure 3).

The observed pattern of sequence homology of 76F14H49 (a subclone of phage 76F14) with other subclones was unexpected. The fragment located on the 5’-end between the EcoRI
and HindIII sites of 76F14H49 was present in subclone 76-1HB3-23, but >3.5 kb away from the preceding sequence in subclone 76F14H49. The same 5′-fragment was also located in the middle part of two other subclones (76-1B3b and 76-1HB2-17, which have 100% sequence homology with each other), whereas in the surrounding sequences no homology was found (Figure 3). A similar situation was found for the 3′-end of subclone 76F14H49 between the HindIII and EcoRI sites. This has large homology with the middle part of a subclone 76-1HB3-24, but not with the surrounding sequences.

This last subclone (76-1HB3-24) was obtained by digestion with HindIII and BamHI and therefore an internal HindIII site was an unexpected finding. Probably, 76-1HB3-24 has originated from two fragments that were ligated to each other during ligation into the vector. This subclone has large sequence homology (>90%) in the 3′-site between the BamHI site and the first HindIII site with subclone 76-1HB1-7. The two subclones 76-1EB1-2 and 76-1HB2-18 had 100% homology in their overlapping area but no sequence homology with other subclones.

Sequence comparison with consigned sequences of the GenBank database revealed some sequence homology between areas of several subclones and the repetitive mouse L1 and rat LINE families.

**Isolation and molecular characterization of the chromosome 4q probe (cos25)**

A PCR product of one of the phages (phage 9.1) isolated by Essers et al. (1995), containing the 60-56 DNA family (see above), was used to screen a rat genomic cosmid library. One of the isolated cosmids (cosmid 25) hybridizes on the q arm of chromosome 4 (4q41-2, Figure 1C) and the fluorescent signal is bright and compact, even in interphase nuclei. In order to determine the repetitive structure of cosmid 25 (cos25), we followed two strategies: Southern blotting and sequence analysis. The Southern blot shows bands located at 1.7 and 0.3 kb after digestion with Sau3A and a band at 2.2 kb after digestion with EcoRI (Figure 4A). In the EcoRI lane smaller bands at 4.4 and 6.6 kb were also visible, indicating a tandem repeat organization. On the basis of this Southern blot it was decided to subclone cos25 with the restriction enzymes EcoRI and Sau3A. The subclones were tested in a FISH experiment for positive signals on chromosome 4 and subsequently sequenced (see Table II). The EcoRI subclone (25E2) is 2174 bp long and contains three Sau3A sites (Figure 4B). The Sau3A subclone 25S6 is 373 bp long and subclone 25S5 is 1676 bp long. Subclone 25S5 contains the EcoRI site and the sequence of 25S5 spans the beginning and end of the EcoRI repeat. On the basis of these sequence data, it can be concluded that the repeat unit of cos25 is a 2174 bp long EcoRI unit that contains three Sau3A sites and is tandemly organized.

Searching for sequence homologies with other known sequences resulted in an interesting similarity with a rat DNA clone of 142 bp containing synaptonemal complex-associated DNA (Pearlman et al., 1992). The sequence similarity (98% homology) is only with the last 50 bp of this rat clone and is located at position 512 of the 25E2 clone and at position 1060 of the 25S5 clone. This sequence homology (although over a very short region) may suggest a possible role of this cos25 repeat family in formation of the synaptonemal complex during meiosis. However, further investigations are needed to confirm this idea.

**Fig. 4.** (A) Southern blot of cos25. The marker is λ×HindIII. n, number of repeat units; E, EcoRI; Sa, Sau3A; R, HindIII. (B) Schematic representation of the tandem repeat of cos25 and the different subclones isolated, 25E2, 25S6 and 25S5.

**Isolation and molecular characterization of the chromosome 19q probe (cos42-47)**

Parallel to the isolation of the 60-56 repeat family, isolation of rat whole chromosome paint probes by bivariate flow sorting followed by degenerate oligonucleotide-primed (DOP) PCR amplification was carried out (Hoebbe et al., 1994). In order to determine if these repeat families were also present on the other chromosomes, flow-sorted chromosomes were amplified with primers specific for the 60-56 family (described in Essers et al., 1995). Results showed that on a large number of flow-sorted chromosomes PCR products were indeed found (data not shown). The PCR product of chromosome 20 was used to screen a rat genomic library and this resulted in the isolation of a number of cosmids, one of which (cos42-47) hybridized on the X chromosome near the centromere (Hoebbe and de Stoppelaar, 1996). Unfortunately, this probe hybridizes on multiple sites along the X chromosome, resulting in multiple fluorescent signals per X chromosome in interphase nuclei after FISH. This makes the probe unsuitable for aneuploidy detection and it was decided not to study this probe further. Instead, we used the insert of this cos42 to screen a cosmid library and this resulted in the isolation of a number of cosmids, one of which (cos42-47) hybridized on the 19q probe (cos42-47).

To determine the molecular structure of cos42-47 it was digested using the restriction enzymes SmaI, Sau3A, BamHI, EcoRI and HindIII. Since very distinct bands of 0.8 and 1.7 kb, respectively, were seen after gel electrophoresis of the SmaI and Sau3A fractions (data not shown), cos42-47 was
subcloned using these two restriction enzymes. Sequencing of subclone 42-47Sma1 (only one strand sequenced) revealed an insert of 843 bp; the Sau3A subclones were only partially sequenced (Table II). A search for homology with consigned sequences of the GenBank database showed sequence homology of our subclones with the rat gene for 5S RNA (Suzuki et al., 1996; Frederiksen et al., 1997). In addition, the chromosomal localization of cos42-47 on 19q12 corresponds with the localization described for the 5S RNA gene (Szabo et al., 1978; Suzuki et al., 1996; Frederiksen et al., 1997). From these results, and from comparisons with the known sequence, we conclude that the repeat unit of cos42-47 consists of tandemly repeated 5S RNA genes and that the Sau3A subclones contain the whole 5S RNA unit (for a schematic representation see Figure 5).

Frederiksen et al. (1997) demonstrated by Southern blotting the presence of 100–200 copies of the 5S RNA gene in the rat genome. If they are all present on chromosome 19q12 a region of at least 170 kb will be stained during FISH. The fluorescent signal of cos42-47 is generally small and compact, in both metaphase and interphase nuclei, indicating that the DNA stays relatively condensed during (almost) the whole cell cycle even though this repeat family contains actively transcribed genes. Besides the active 5S RNA gene, an inactive pseudo-5S RNA gene has also been isolated by Frederiksen et al. (1997). The pseudogene is located on chromosome 12q12 and the number of repeats is 150–250, with a copy length of 2.5 kb. As far as we know, this probe is not used for aneuploidy detection but it is probably a very good candidate. The 5S RNA gene families are also present in the genome of other species, making it worthwhile to examine to what extent 5S RNA genes can be used as probes for aneuploidy detection in these species.

Detection of aneuploidy using the isolated probes

In order to test the isolated probes for their suitability for detection of aneuploidy, we performed two series of dual colour FISH on slides from a previously described in vitro cytokinesis-blocked micronucleus assay with DES in rat fibroblasts (see de Stoppelaar et al., 1997). On these slides, the frequency of binucleated cells containing one or more micronuclei (MN) was increased after DES treatment from an average of 1.8% in controls to an average of 3.8 and 8.1% in the 10 and 13.5 μg/ml cultures, respectively (Table III). The observed MN frequencies were similar to those found on the propidium iodide stained slides (hybridized with the centromeric probe) scored previously (de Stoppelaar et al., 1997).

Dual colour FISH with probes cos76-1 (19p) and 25S5 (4q): all binucleated cells

After dual colour FISH with probes cos76-1 (19p) and 25S5 (4q, a subclone of cos25) the distribution of hybridization signals among the two daughter nuclei (and MN) of the binucleated cells was determined. For evaluation of non-disjunction and chromosome loss, only those binucleated cells that contained four hybridization signals of the respective probe were taken into account. Cells with three hybridization signals in one nucleus and one in the other were scored as cells with non-disjunction (Figure 1E) and the presence of a hybridization signal in the MN was scored as chromosome loss (Figure 1F). Treatment of the cells with 10 or 13.5 μg/ml DES resulted in induction of non-disjunction and chromosome loss, regardless of which probe was scored (Table IV).

The absolute frequencies were low but the induction was clearly present, especially in the high dose group. Total malsegregation frequency (non-disjunction + chromosome loss) of chromosomes 19 and 4 was increased by at least 10-fold due to DES treatment (0.3 in control versus 0.35 in the high dose group) and was statistically significant in the high dose group (middle dose group borderline significant).

Dual colour FISH with probes cos76-1 (19p) and 25S5 (4q): micronucleated binucleated cells

In order to obtain more information on chromosome segregation in micronucleated binucleated cells (MNBC), additional cells containing MN were scored on the slides hybridized with cos76-1 and 25S5 (4q and 19p). Many different distributions of hybridization signals were observed in the micronucleated cells, the main category being the normal distribution of two signals in each nucleus and none in the MN (Table V). In the treated cultures, the percentage of MNBC with malsegregated chromosomes (non-disjunction or signals in the MN) was increased compared with control cultures. Non-disjunction of chromosome 19 increased from 0.7% in control cultures to 2.4% in the high dose group and with 25S5 from 0.3% in controls to 0.9% in the treated cultures (Table V, column 9). The non-disjunction frequencies in the MNBC were consistently higher than in binucleated cells without MN (data not shown), but this difference was not significant. More studies with a larger number of micronucleated cells are needed to determine whether this effect reflects the actual situation or is coincidental.

With respect to chromosome loss induced by aneugens, a concentration-dependent effect is expected: chromatid loss (one MN with one signal) occurring at lower doses or at higher frequencies than chromosome loss (one MN with two signals) or double chromatid loss (two MN with one signal each). With the chromosome 19 probe this concentration effect was indeed observed (Table V, columns 5–7). The frequency of cells with chromatid loss measured with cos76-1 increased from 0.9% in control cultures to 5.4% in the highest dose group (Table V, column 5). Chromosome loss or double chromatid loss was not observed in control cultures and was 1.7 and 2.3% in the 10 and 13.5 μg/ml DES cultures, respectively (Table V, columns 6 + 7).

With probe 25S5, chromatid loss again occurred more frequently than chromosome loss, but dose dependency was not observed (Table V, columns 5–7). This was mainly due to the high frequencies of MN containing hybridization signals in the control cultures. This is also illustrated in Figure 6, showing a comparison of the number of hybridization signals
in MN detected with the chromosome 4 or 19 probe with the general centromeric probe 18-5 (rat satellite I DNA). This general centromeric probe hybridizes on the centromeres of 17 of the 21 chromosome pairs (not on chromosomes 1, 19, 20, X and Y; see de Stoppelaar et al., 1997) and is used to detect chromosome loss in general. The comparison shows that the signal of the chromosome 4 probe is clearly over-represented in MN of the control cultures (Figure 6). A possible explanation is that some of the observed MN with signals in the control cultures contain acentric fragments of chromosome 4 instead of whole chromosomes, which is not unlikely considering the relatively large size of chromosome 4. Due to the position of the probe on 4q4 (Figure 1C), a majority of these chromosome/chromatid breaks will be detected using this probe (in addition to chromosome loss). In the treated cells there may have been a shift in the fraction of MN originating from chromosome breaks to MN originating from chromosome loss, since the total frequency of MN with hybridization signals was not increased. However, in the general cell population (binucleated cells and micronucleated binucleated cells) chromosome loss did increase after DES treatment (Table IV), due to the increased MN frequency in these cells.

Table IV. Non-disjunction and chromosome loss in binucleated rat fibroblasts, detected with probes 25S5 (4q) and cos76-1 (19p).

<table>
<thead>
<tr>
<th>DES (µg/ml)</th>
<th>Chromosome 19 (cos76-1)</th>
<th>Chromosome 4 (25S5)</th>
<th>Total malsegregation of chromosomes 4 and 19 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total BC scored (%)</td>
<td>Non-disjunction (%)</td>
<td>Chromosome loss (%)</td>
</tr>
<tr>
<td>0</td>
<td>1263</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>1241</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>10</td>
<td>1302</td>
<td>0.6</td>
<td>0.2</td>
</tr>
<tr>
<td>10</td>
<td>1235</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>13.5</td>
<td>1286</td>
<td>1.8</td>
<td>0.3</td>
</tr>
<tr>
<td>13.5</td>
<td>1202</td>
<td>1.1</td>
<td>0.6</td>
</tr>
<tr>
<td>13.5</td>
<td>1241</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>13.5</td>
<td>1235</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>13.5</td>
<td>1286</td>
<td>1.8</td>
<td>0.3</td>
</tr>
<tr>
<td>13.5</td>
<td>1202</td>
<td>1.1</td>
<td>0.6</td>
</tr>
</tbody>
</table>

BC, binucleated cells.

The availability of two probes for chromosome 19, hybridizing on either side of the centromere, gives us the opportunity to distinguish aneugenic from clastogenic events, which is illustrated by the results of a second series of dual colour FISH using cos76-1 (19p) and cos42-47 (19q) as probes. Since both these probes hybridize on the same chromosome, they should segregate simultaneously among the two nuclei of the binucleated cells. Thus, non-disjunction will result in three signals for the two probes in one nucleus and one in the other, whereas a signal for both probes simultaneously in the MN indicates chromosome loss. As in the first hybridization series, non-disjunction (3-1/3-1) of chromosome 19 was significantly induced in the cultures treated with 13.5 µg/ml DES (Table VI). Chromosome loss of chromosome 19 was not observed in control cultures and increased to an average of 0.3 and 1.4% in the 10 and 13.5 µg/ml cultures, respectively (Table VI).

Apart from non-disjunction and chromosome loss, unequal segregation patterns of the probes were also observed, for example a 2-2 distribution of one probe and a 3-1 distribution of the other (Table VI). This indicates that cytogenetic events other than chromosome loss or non-disjunction occur, e.g. breaks or translocations. This unequal segregation of hybridization signals can arise in a cell harbouring a translocation involving one of the arms of chromosome 19. Depending on the segregation of the translocation chromosome, a 2-2/2-2 or 2-2/3-1 distribution will arise in the binucleated cell. On the other hand, a 2-2/3-1 distribution may have originated from incorporation of an acentric fragment of chromosome 19 into one of the nuclei or by fusion of a MN containing a hybridization signal with one of the main nuclei. The frequency of these cells with breaks or translocations was induced by treatment of the cells with DES (~2.5-fold increase), but to a lesser extent than the induction of non-disjunction (~10-fold increase; Table VI).

Thus, the present results demonstrate that DES has a predominantly aneugenic mode of action, which is known to be the consequence of inhibition of microtubule assembly during mitosis (Parry, E.M. et al., 1982; Sakakibara et al., 1991; Oda et al., 1995), and that DES can also act as a clastogenic agent (reviewed by Marselos and Tomatis, 1993). The results also demonstrate that using a single probe for chromosome 19 to detect non-disjunction would result in an over-estimation of the actual non-disjunction frequency (Table VI, columns 6 and 7 compared with column 4). This was most evident with the 19p probe cos76-1. On the basis of these results the use of both chromosome 19 probes simultaneously for the detection of aneuploidy is recommended.

Cells with other distributions and polyploid cells

Besides cells with four hybridization signals per probe, cells with more or less than four signals were also observed. The frequencies were in the range 10–25% and varied per probe. The lowest frequency of cells with other than four signals was observed with probe 25S5 (4q) and was ~10% (data not shown) and the highest frequency was scored with probe cos76-1 (19p,
Table V. Frequency of micronucleated binucleated cells with the indicated distributions of hybridization signals of cos76-1 (chromosome 19p) and 25S5 (chromosome 4q)

<table>
<thead>
<tr>
<th>DES (µg/ml)</th>
<th>Total scored</th>
<th>Normal distribution (%)</th>
<th>Non-disjunction (%)</th>
<th>Chromatid loss (%)</th>
<th>Chromosome loss or double chromatid loss (%)</th>
<th>Non-disjunction + loss (%)</th>
<th>Total non-disjunction (column 4 + 8) + loss (%)</th>
<th>Total chromosome loss (column 5–8) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe cos76-1 (chromosome 19p)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>479</td>
<td>98.3</td>
<td>0.7</td>
<td>0.9</td>
<td>0.0</td>
<td>0.0</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>10</td>
<td>545</td>
<td>93.0</td>
<td>0.8</td>
<td>4.6</td>
<td>0.3</td>
<td>1.4</td>
<td>0.0</td>
<td>0.8</td>
</tr>
<tr>
<td>13.5</td>
<td>669</td>
<td>89.9</td>
<td>2.1</td>
<td>5.4</td>
<td>0.2</td>
<td>2.1</td>
<td>0.3</td>
<td>2.4</td>
</tr>
<tr>
<td>Probe 25S5 (chromosome 4q)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>547</td>
<td>95.2</td>
<td>0.2</td>
<td>3.2</td>
<td>0.0</td>
<td>1.4</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>10</td>
<td>634</td>
<td>94.5</td>
<td>1.0</td>
<td>3.8</td>
<td>0.3</td>
<td>0.7</td>
<td>0.1</td>
<td>1.1</td>
</tr>
<tr>
<td>13.5</td>
<td>792</td>
<td>94.0</td>
<td>0.9</td>
<td>4.1</td>
<td>0.0</td>
<td>0.9</td>
<td>0.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>

The frequencies represent the total of two duplicate cultures, which in turn are calculated as the average of two scorers.
The only category of cells with other than four signals that was influenced by treatment with DES was polyploid cells. The frequency of binucleated cells with seven or eight hybridization signals per probe (the latter likely representing polyploid cells with overlapping signals) was decreased in the treated cultures, irrespective of which probe was used (Table VII). This decrease in polyploid binucleated cells was also observed in in vivo and in vitro MN assays with carbenzadim in the same cells (de Stoppelaar et al., this issue). Polyploidy in binucleated cells can only arise by nuclear division in a cell that was already polyploid before addition of cytochalasin B to the medium. Alternatively, polyploid binucleated cells may arise when binucleated cells undergo a second, unsuccessful, nuclear division in the presence of cytochalasin B, forming two polyploid nuclei instead of four diploid nuclei by, for example, nuclear fusion. In both cases, the toxicity of DES may be a possible explanation of the observed decrease. Polyploid cells may be more sensitive to the toxic effects of DES than diploid cells and have less chance of surviving the treatment. Alternatively, the cell cycle delay induced by DES (determined by scoring the percentage of binucleated cells and reported in de Stoppelaar et al., 1997) will decrease the chance that the cells undergo a second nuclear division in the presence of cytochalasin B, thereby decreasing the frequency of polyploid binucleated cells in DES-treated cultures.

**Evaluation of the usefulness of the isolated probes for the detection of aneuploidy**

We have tested the isolated probes for their usefulness for the detection of aneuploidy by means of FISH on slides of an in vitro MN assay with DES. With all three probes a DES-induced increase in non-disjunction and chromosome loss could be detected, indicating their usefulness for detection of aneuploidy. Nevertheless, the probes have clearly different hybridization characteristics.

Due to the tandem organization of the repeat unit, the hybridization signal of cos25 (and subclone 25S5, 4q) is very bright and distinct in interphase nuclei (Figure 1C), which enables an accurate determination of the number of signals in a nucleus. This was illustrated by the fact that on the MN slides the percentage of cells showing other than four signals for 25S5 (4q) was lower than with the other two probes. A clear increase in non-disjunction and chromosome loss was observed with this probe in the DES-treated cultures (Table IV), showing that this probe is very suitable for detection of
aneuploidy. However, the probe is located on the long arm of the chromosome (4q41-2), closer to the telomere than to the centromere (Figure 1C). Consequently, in the case of a chromosome break between the hybridization region and the centromere, the signal will be lost from the main nucleus and end up in the MN. Thus, the presence of a hybridization signal in the MN can be indicative of either an aneugenic (chromosome loss) or a clastogenic (chromosome break) effect, making the probe less suitable for accurate detection of chromosome loss (see above).

The two probes for chromosome 19, cos76-1 and cos42-47, are both useful for aneuploidy detection, as illustrated by the observed increase in non-disjunction and chromosome loss in the DES-treated cultures (Table VI), but the hybridization signals of the probes differ from each other. cos42-47 (19q) shows a small but distinct signal in interphase nuclei, facilitating scoring of this probe. As with most probes that are located on a chromosome arm, ‘split’ signals were sometimes observed, which are likely to represent G2 cells. The FISH signals of Environmental Programme (contract no. STEP-CT91-0159).

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reading this manuscript, Henny Verharen and Dr Jan van Benthem for isolating and scoring of this probe. As with most probes that are located (chromosome loss) or a clastogenic (chromosome break) effect, making the probe less suitable for accurate detection of chromosome loss.

The fact that the highest number of cells with other than four hybridization signals were observed with probe cos76-1 (19p), in particular cells with the 2-1 distribution of signals. These problems of scoring possible artefacts can be overcome by evaluating only those cells containing four hybridization signals for the probe. Due to the compactness of the signal of cos42-47 (19q) compared with the signal of cos76-1 (19p), the use of cos42-47 for the detection of chromosome 19 is preferred. However, the use of both probes for chromosome 19 in combination in a dual colour FISH is the recommended option, since this allows the simultaneous detection of non-disjunction, chromosome loss and other events, such as chromosome breaks. Considering that most aneugens also have (slight) clastogenic effects and vice versa, this methodology adds to the sensitivity of the assay.

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