Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 4442–4447, April 1996
Evolution

A methylated human 9-kb repetitive sequence on acrocentric chromosomes is homologous to a subtelomeric repeat in chimpanzees

(genomic scanning/repetitive DNA/subtelomeric sequences/DNA methylation/evolution)

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Contributed by J. V. Neel, January 24, 1996

ABSTRACT **We have implemented an approach for the detection of DNA alterations in cancer by means of computerized analysis of end-labeled genomic fragments, separated in two dimensions. Analysis of two-dimensional patterns of neuroblastoma tumors, prepared by first digesting DNA with the methylation-sensitive restriction enzyme *Not* I, yielded a multicopy fragment which was detected in some tumor patterns but not in normal controls. Cloning and sequencing of the fragment, isolated from two-dimensional gels, yielded a sequence with a strong homology to a subtelomeric sequence in chimpanzees and which was previously reported to be undetectable in humans. Fluorescence *in situ* hybridization indicated the occurrence of this sequence in normal tissue, for the most part in the satellite regions of acrocentric chromosomes. A product containing this sequence was obtained by telomere-anchored PCR using as a primer an oligonucleotide sequence from the cloned fragment. Our data suggest demethylation of cytosines at the cloned *Not* I site and in neighboring DNA in some tumors, compared with normal tissue, and suggest a greater similarity between human and chimpanzee subtelomeric sequences than was previously reported.**

Human chromosomes possess at their extremities TTAGGG telomeric repeats which protect the chromosomes from degradation or instability (1). This repetitive sequence is distal to a complex of subterminal repetitive sequences observed at many telomeres (2–4). A high level of sequence divergence between copies of the repetitive sequences suggests that the sequence family is ancient (5). The complex of subtelomeric sequences can differ from chromosome to chromosome, and some of the subtelomeric sequences can additionally be located elsewhere along chromosomes. Some evidence suggests the possibility of exchange of telomeres and subtelomeric sequences between nonhomologous chromosome ends (6).

The use of a telomere-anchored PCR strategy has allowed isolation of DNA corresponding to several subterminal sequences and has provided the means to compare subtelomeric sequences between humans and chimpanzees (7). A major difference between the karyotypes of humans and African apes which may be attributable to differences in subtelomeric sequences is the presence of positively staining G bands at the ends of many chromosome arms in the chimpanzee and gorilla but absent from human chromosomes (8). Using a telomere-anchored PCR strategy, Jeffreys and colleagues (7) reported the occurrence of subtelomeric sequences in the human and chimpanzee genomes that were distinct to each species. In particular, a 118-bp sequence was found in the chimpanzee and was not detected in the human or orangutan genomes (7). Additionally, a PCR product, composed of a 32-bp repeat, was obtained from the chimpanzee which hybridizes to terminal bands in chimpanzees and gorillas and which was not detected in humans or orangutans, suggesting that the organization of sequences adjacent to telomeres is very different between certain primates. This reported difference is among the most striking yet observed between the two species.

We have implemented a computer-based approach for the analysis of restriction fragments of human genomic DNA, separated by two-dimensional (2-D) electrophoresis (9). Analysis of the 2-D patterns has resulted in a high yield of restriction fragment length polymorphisms among approximately 2000 end-labeled fragments simultaneously visualized (10). We are also currently utilizing this approach to detect amplifications, deletions, and methylation changes in genomic DNA from malignant tumors relative to nonmalignant tissue from the same individuals. In a study of neuroblastomas, we observed a fragment which occurred in a high copy number in three tumors relative to peripheral blood lymphocytes from the same patients. Cloning, sequencing, and further characterization of this fragment indicated a strong homology with one of the subtelomeric sequences which was reported to occur in chimpanzees but which was undetectable in humans (7). Our data suggest that this subtelomeric sequence is subject to methylation in some normal tissues and suggest a greater similarity between human and chimpanzee subtelomeric sequences than previously recognized.

MATERIALS AND METHODS

DNA Samples. Genomic DNA was obtained from tumor tissue, peripheral blood lymphocytes and Epstein–Barr virus-transformed lymphoid cell lines, and other normal tissues from patients with cancer. High molecular weight DNA was extracted from cell nuclei of 2×10^7 cells. After phenol extraction of the proteinase K digest, the DNA was precipitated with ethanol and dissolved in TE buffer (10 mM Tris·HCl/1 mM EDTA, pH 8.0). The concentration of DNA was adjusted to 300 ng/ml.

Preparation of the 2-D Gels. Gels were prepared as previously described (9). Briefly, genomic DNA was digested with *Not* I and *Eco*RV restriction enzymes and the *Not* I-derived 5′ protruding ends were ^{32}P-labeled. The fragments were electrophoretically separated in agarose disc gels, which were subsequently treated with *Hin*fI to further cleave the fragments

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Abbreviations: 2-D, two-dimensional; FISH, fluorescence *in situ* hybridization.

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**The sequence reported in this paper has been deposited in the GenBank data base (accession no. U53226).

in situ. The resulting fragments were separated perpendicularly in a 5.25% polyacrylamide gel (33 cm × 46 cm × 0.08 cm). Gels were dried on filter paper at 80°C and exposed to PhosphorImager plates (Molecular Dynamics) at room temperature for 5 days. Images were obtained after scanning of the PhosphorImager plate.

Cloning of the DNA Fragment. For cloning of the DNA fragment, preparative gels were used. A total of 1 μg of radiolabeled DNA mixed with 2 μg of nonradiolabeled DNA was loaded on the top of the first-dimensional agarose gel. After the second-dimension separation in polyacrylamide, the gel was exposed without drying to x-ray film (RX; Fuji) for 24 h at −80°C. The *Not* I–*Hin*fI fragment corresponding to the DNA spot of interest was recovered from the polyacrylamide gel, resuspended in TE buffer containing tRNA, bromophenol blue, and xylene cyanol before purification by electrophoresis onto DEAE-cellulose membrane (11, 12). After electrophoresis, the DEAE-cellulose membrane was washed with a low-salt wash buffer (50 mM Tris·HCl, pH 8.0/0.15 M NaCl/10 mM EDTA) and the DNA was recovered by elution with a high-salt elution buffer (50 mM Tris·HCl, pH 8.0/1 M NaCl/10 mM EDTA). Following this step, the DNA was extracted twice with phenol and precipitated overnight at −20°C after addition of 3 vol of absolute ethanol. The DNA was then recovered by centrifugation, washed with 70% ethanol, and resuspended in water. Following this step, the DNA was ligated in a modified vector SK (+) pBluescript (*Not* I–*Pst* I-digested pBluescript with oligonucleotides ligated to the *Pst* I protruding end to generate a *Hin*fI cohesive end) in the presence of DNA ligase at 16°C for 40 h. The transformation was performed by electroporation using the Epicurian Coli XL1-Blue MRF′ electroporation-competent cells (Stratagene). After selection of the clones containing the insert, sequencing was performed on double-stranded DNA by the dideoxynucleotide chain-termination method, using a sequencing kit (United States Biochemical).

Southern Blot Analysis. Genomic DNA was digested to completion with *Eco*RI, *Not* I, *Not* I/*Eco*RV, *Msp* I, or *Hpa* II and electrophoresed on a 1.0% agarose gel. The DNA was then transferred onto nylon membranes (Hybond N+, Amersham) according to the protocol of the manufacturer and hybridized in 50% formamide at 42°C, using the ^{32}P-labeled cloned fragment as a probe. After overnight hybridization, the membranes were washed at high stringency with a final wash in 0.2× SSC/0.5% SDS at 65°C for 30 min (1× SSC = 150 nM NaCl/15 mM sodium citrate, pH 7.0). The membranes were air dried and visualized by PhosphorImager technology. The same procedure (except the final wash at 60°C was only for 30 min) was followed for hybridization of a zooblot membrane containing 5 μg of *Xba* I-digested genomic DNA per lane from seven eukaryotic species: human, chimpanzee, baboon (*Papio ursinus*), fish (*Oncorhyrchus mykiss*), nematode (*Caenorhabditis elegans*), *Drosophila melanogaster*, and yeast (*Saccharomyces cerevisiae*).

Fluorescence *in Situ* Hybridization (FISH). FISH on R-banded metaphase chromosomes from a normal woman was performed by a modification of the protocols of Lemieux *et al.* (13), Pinkel *et al.* (14), and Lichter *et al.* (15). In brief, metaphase chromosomes were prepared from peripheral blood lymphocytes after overnight synchronization with 5-bromodeoxyuridine and thymidine release. Cells were harvested and slides were prepared according to standard cytogenetic techniques prior to hybridization with the biotinylated fragment of interest.

PCR Conditions. PCR amplifications between the repetitive sequence and the telomere were carried out essentially as described (4) in a 20-μl volume using 0.2 μl of *Taq* DNA polymerase. The reaction mixtures included 1 μM primer TelD (5′-GGCCATCGATGAATTCCTCACCCTCACCCTCACCCTCA-3′) and 1 μM primer which hybridizes in the cloned part of the repetitive sequence (5′-TCCGCGAAGATCTGAGTACAG-3′) and were cycled at 94°C for 30 sec, 69°C for 3 sec, and 72°C for 5 min for 35 cycles. The PCR product was cloned in the pCRII vector (Invitrogen Corporation) prior to restriction enzyme and sequence analysis.

RESULTS

A Novel Human Repetitive DNA Sequence. Genomic DNA was isolated from 10 neuroblastomas, 9 lung tumors, 20 brain tumors, and 7 esophageal tumors and from normal tissues from the same patients—i.e., peripheral blood lymphocytes and normal lung, brain, and esophagus, respectively, for the different tumor types. For most tumors, comparison of the 2-D patterns of tumor and normal tissue, obtained by cleaving DNA with *Not* I, *Eco*RV, and *Hin*fI, revealed the presence of one or more multicopy fragments in the tumor pattern that were either absent for some fragments or present at an intensity suggestive of two-copy fragments for others (Fig. 1). One multicopy fragment was observed in three neuroblastoma tumors and one lung tumor and was absent from genomic DNA from normal tissue from the same patients (Fig. 1). The size of the *Not* I–*Eco*RV fragment separated in the first dimension, from which the *Not* I–*Hin*fI fragment was derived, was estimated from the position of the *Not* I–*Hin*fI fragment in the horizontal axis to be 3.0 kb. The size of the *Not* I–*Hin*fI fragment was estimated to be 600 bp, on the basis of its migration in the second dimension. Its intensity was approximately 20 times greater than that observed for most of the spots in the patterns which consisted of two copy fragments.

For cloning of the *Not* I–*Hin*fI fragment, DNA was recovered from a preparative gel containing a mixture of radiolabeled and unlabeled DNA from a neuroblastoma tumor which displayed the multicopy fragment. The recovered DNA was cloned into a modified SK (+) pBluescript vector. Eight clones containing the *Not* I–*Hin*fI DNA fragment were sequenced. All clones yielded a 580-bp *Not* I–*Hin*fI DNA sequence when the T3 and T7 promoter primer were used (accession no. U53226). Comparison of the different sequences revealed single base differences in the sequence between the eight clones: the sequences from four clones were identical, whereas the remaining four clones had substitutions (clone 122T/23, A → G base 57 and TT → GG bases 548 and 549; clone C2/7, A → T base 395; clone C2/5, G → A base 381; and clone C2/12, T → G base 460). A homology search uncovered a 115-bp subtelomeric sequence isolated from the chimpanzee by a telomere-anchored PCR strategy (accession no. X74282) (7) which exhibited almost complete identity with a portion of the sequence we have determined.

Methylation Status of the *Not* I Site in the Repetitive DNA Sequence. Using the cloned fragment as a probe, we performed Southern blotting with genomic DNA from different tumors and corresponding normal tissue. DNA digested with *Eco*RI, yielded a DNA band of 6 kb, of equal intensity in normal tissue and in tumor tissue (data not shown). Digestion of neuroblastoma tumor and peripheral blood lymphocyte DNA from the same patient with *Not* I, which is a methylation-sensitive restriction enzyme, yielded a major large band in both samples. A second, smaller band (9 kb) with less intensity was detected in the lane containing digested genomic DNA from the tumor but not in the lane containing digested genomic DNA from peripheral blood lymphocytes (Fig. 2). This 9-kb fragment represents the entire repetitive DNA sequence. Likewise, when the same DNAs were double-digested with *Not* I–*Eco*RV, a major band was detected in tumor and control DNA and a second band with less intensity was detected in digested genomic DNA from the tumor but not from peripheral blood lymphocytes. The major band corresponds to a DNA fragment of 9 kb and the second band corresponds to a DNA fragment of 3 kb (Fig. 2). These results clearly suggest

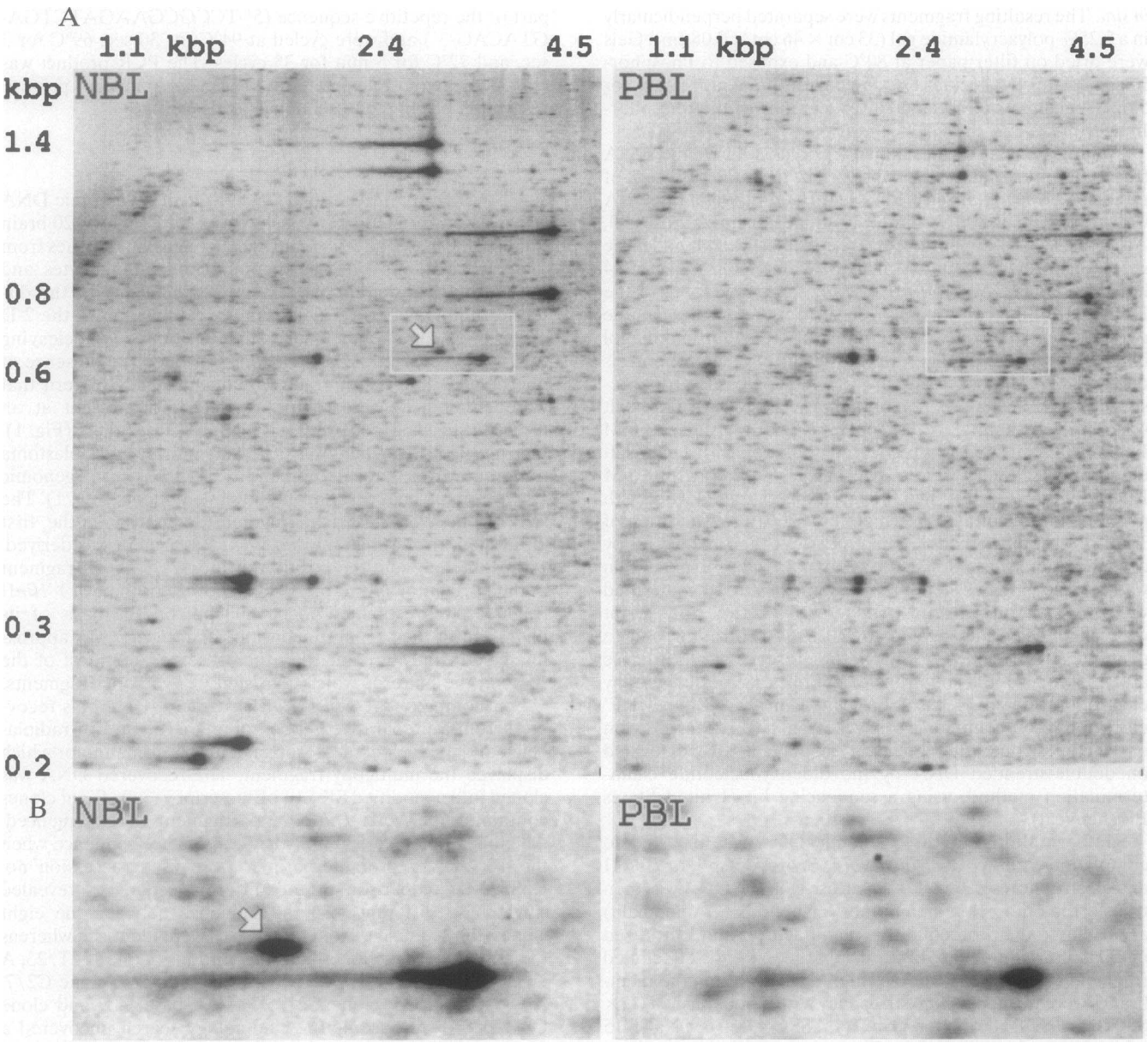

FIG. 1. Detection of multicopy fragments by 2-D analysis of genomic digests. (*A*) Digital image of 2-D patterns of neuroblastoma (NBL) tumor DNA and of control tissue DNA (peripheral blood lymphocytes) (PBL) from the same patient. Fragment sizes for each dimension are indicated. (*B*) Close-up of a region containing the multicopy fragments (boxes in *A*).

that the detection of a multicopy fragment in 2-D DNA gels of the neuroblastoma tumor was not the result of an amplification of a fragment which occurs in two copies in normal tissue. The Southern blot showed that the *Not* I recognition site was methylated in the peripheral blood lymphocytes and resistant to digestion with the methylation-sensitive restriction enzyme *Not* I. In contrast, in the tumor tissue, the *Not* I recognition site responsible for the presence of this genomic fragment in the tumor pattern was demethylated in a fraction of the repetitive DNA sequence or in a fraction of the tumor cells and thus yielded a multicopy fragment in the gel.

Methylation at Additional Sites. Genomic DNA from a tumor which yielded the multicopy fragment and genomic DNA from two controls were digested with the isoschizomer pair of restriction enzymes *Msp* I and *Hpa* I (*Hpa* I does not cut when the restriction site CCGG is methylated). DNA digested with *Msp* I yielded three DNA bands at 0.7 kb, 0.4 kb, and 280 bp, of equal intensity in the three samples, following hybridization of the Southern blot with the cloned fragment as a probe (Fig. 3). DNA digested with *Hpa* II yielded the three bands in the case of tumor DNA. These bands were considerably reduced or absent in control DNA, in which a ladder consisting of larger fragments predominated. This result indicates that altered methylation in this repeat sequence in the tumor is not limited to the *Not* I site.

Homology with Other Species. A probe corresponding to the DNA fragment of interest was hybridized to genomic DNA from different eukaryotic species (human, chimpanzee, baboon, fish, nematode, *Drosophila*, and yeast) (Fig. 4). The probe hybridized with the human, chimpanzee, and baboon DNA, not with other species. A band corresponding to a DNA fragment of 9 kb was observed in the human. A band in the same approximate position was observed in the chimpanzee and the baboon. Two additional bands were also observed in chimpanzee and baboon DNA. These two bands correspond to DNA fragments of 27 and 7.5 kb. The results of this hybridization suggest a closer conservation between chimpanzee and baboon genomic DNA than between chimpanzee and human.

Mapping of the Repetitive DNA Sequence. A *Not* I–*Hin*fI DNA fragment corresponding to fragment I was hybridized to

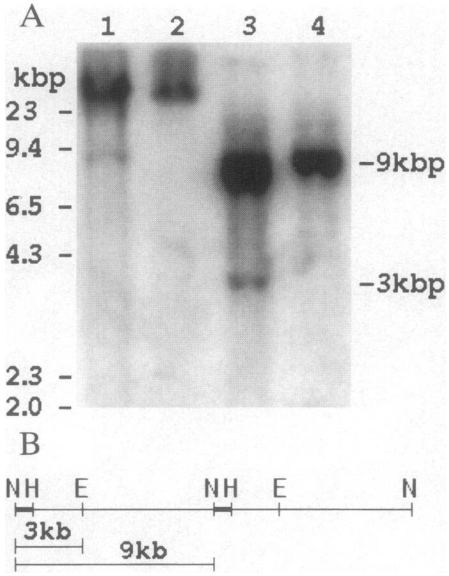

FIG. 2. (*A*) Southern blot analysis using the cloned fragment as a probe. Genomic DNA samples were digested with *Not* I alone (lanes 1 and 2) or *Not* I plus *Eco*RV (lanes 3 and 4); neuroblastoma DNA is in lanes 1 and 3, and peripheral blood lymphocyte DNA is in lanes 2 and 4. Size markers were bacteriophage λ DNA digested with *Hin*dIII. (*B*) Schematic representation of the restriction map of the repetitive DNA sequence. The sites for restriction enzymes used to digest DNA for 2-D gel electrophoresis are shown on the map. The location of the *Not* I–*Hin*fI fragment used as a probe for the one-dimensional Southern blot is shown in bold. N, *Not* I; E, *Eco*RV; H, *Hin*fI.

bromodeoxyuridine-treated normal female human metaphase chromosomes by using FISH (13–15). The analysis, performed on G-banded chromosomes for chromosomal localization, revealed bright, consistent hybridization signals on the satellite regions, on all D-group and G-group chromosomes (chromosomes 13, 14, 15, 21, and 22) (Fig. 5). Consistent signals were also seen in the centromeric regions of chromosomes 3 and 4, specifically, 3q11–12 and 4p11–12 (Fig. 5).

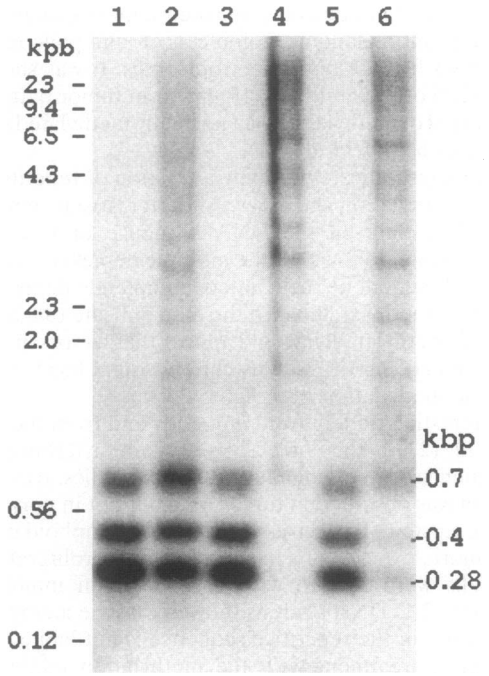

FIG. 3. Methylation status of the repetitive sequence in normal and in tumor tissue, based on Southern blotting analysis using the cloned fragment as a probe. Genomic DNA was digested with *Msp* I (lanes 1, 3, and 5) or *Hpa* II (lanes 2, 4, and 6); neuroblastoma DNA in lanes 1 and 2, normal control no. 1 DNA in lanes 3 and 4, and normal control no. 2 DNA in lanes 5 and 6.

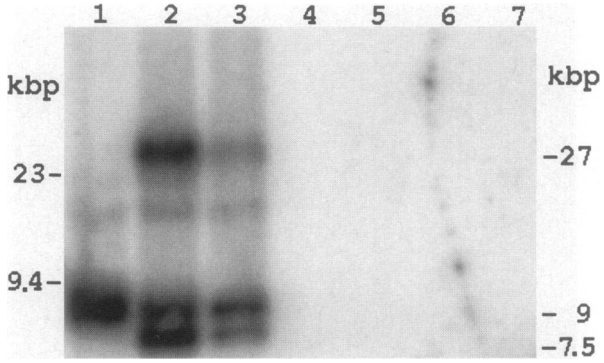

FIG. 4. Southern blotting analysis of DNA from eukaryotic species, using the cloned fragment as a probe. The close-up shown includes all bands detected. Genomic DNA was digested with *Xba* I: lane 1, human; lane 2, chimpanzee; lane 3, baboon; lane 4, fish; lane 5, nematode; lane 6, *Drosophila*; and lane 7, yeast.

Telomere-Anchored PCR Yields the Repetitive DNA Sequence. PCR amplification was performed to determine if the location of the repetitive sequence was subtelomeric as found in the chimpanzee (7). Using a telomeric primer together with a primer derived from the repetitive sequence, we obtained a PCR product that was cloned in the pCR II vector and that yielded an identical sequence for eight clones. This sequence started at base 25 of the cloned repetitive sequence and ended with a variable number of telomeric repeats; the junction between the repetitive sequence and the telomere occurred at the same site for all clones analyzed (at position 389 of the repetitive sequence). The fact that the repetitive sequence was identical for all clones would suggest that the PCR product is amplified from only one repetitive sequence at a subtelomeric location, presumably corresponding to one of the five acrocentric chromosomes. This same 5′ → 3′ sequence orientation occurred in humans as described for chimpanzees (7).

DISCUSSION

Molecular data indicate a strong identity (>98%) between the genomes of humans and chimpanzees. These two primates are grouped in the same subtribe Hominina, with gorillas in tribe Hominini and orangutans in subfamily Homininae (16). Thus humans and chimpanzees are closer to each other than they are to the gorillas. One of the significant differences between the karyotypes of humans and chimpanzees concerns the presence of positively staining G-bands at the ends of many chromosome arms in the chimpanzees but absent from the human chromosomes. It has been suggested than this difference could be related to differences in subtelomeric sequences immediately adjacent to telomeres.

Several human subtelomeric sequences have been isolated, from a specialized plasmid library (2), or by rescuing human telomeres ligated to yeast artificial chromosome (YAC) vectors in yeast cells (17–20). By developing a telomere-anchored PCR strategy, Royle *et al.* were able to determine several new subtelomeric sequences in the human genome (4) and the chimpanzee genome (7). Two of the subterminal sequence families found in the human genome have also been detected in the chimpanzee. However, these sequences were not adjacent to the telomeres in the chimpanzee genome. The subtelomeric sequences found in the chimpanzee were separated into four groups: the first group contained highly repetitive *Alu*- and LI-like elements; clones in the second and third groups were composed of sequences which did not contain repeat structures; and the members of the fourth group were

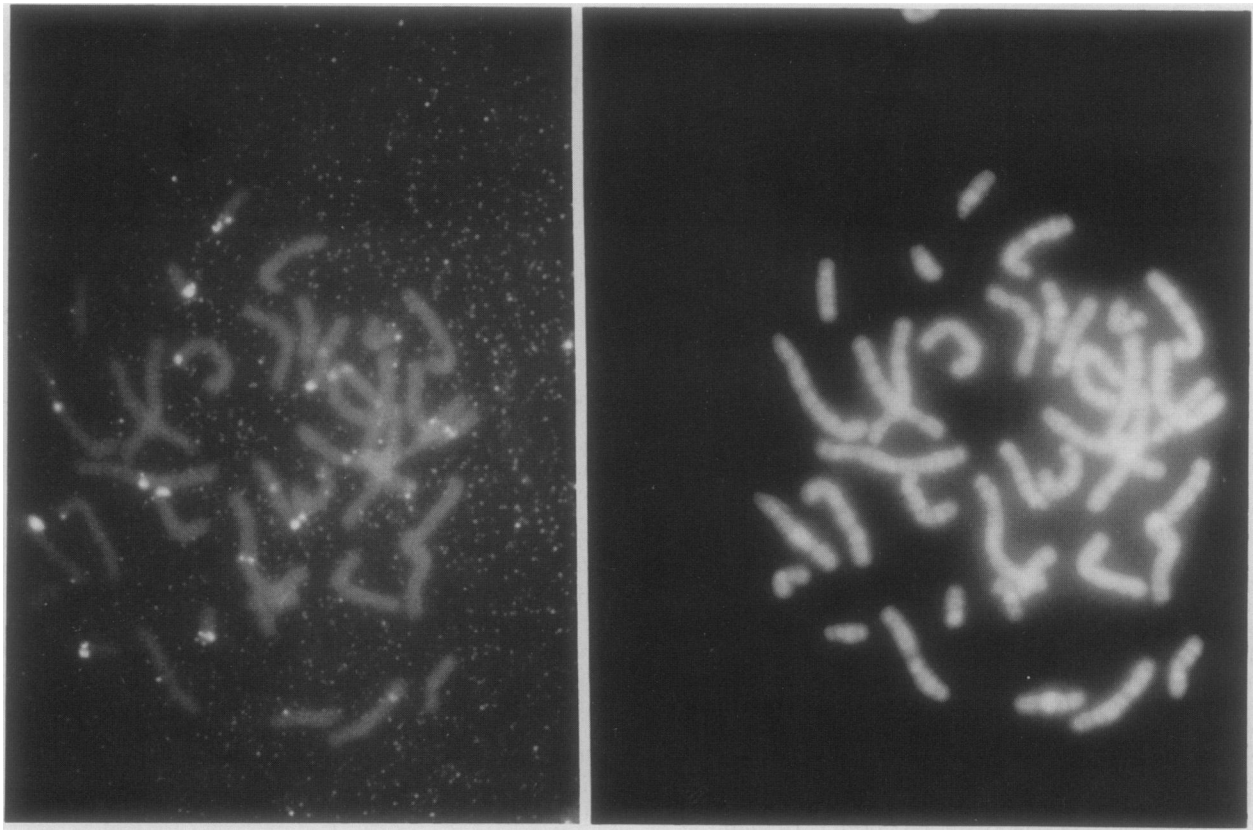

FIG. 5. FISH. An R-banded metaphase cell (*Right*) is shown after *in situ* hybridization with the cloned fragment. (*Left*) A hybridization signal is seen on the satellite regions of all D-group and G-group chromosomes and also in the centromeric regions of chromosomes 3 and 4: 3q11–12 and 4p11–12.

composed of repeat sequences. None of the sequence families from the last three groups were found to exhibit homology with human telomere junction sequences. One of the sequences was composed of 32-bp repeats and hybridized to terminal bands in gorillas but was not detectable in humans or orangutans. Also, a sequence from the second group, a 118-bp segment, was found in chimpanzees but not in humans.

In this study of human genomic DNA from tumors and normal tissue, we have cloned a fragment whose sequence had a strong identity with the 115-bp subtelomeric sequence in the second chimpanzee group. This sequence appears to be part of a highly repetitive sequence of about 9 kb. On the basis of our FISH analysis, this sequence is located on the satellites of all acrocentric chromosomes (chromosomes 13, 14, 15, 21, and 22) and in the pericentromeric region of chromosomes 3 and 4. Furthermore, we were able to generate a PCR product from total human DNA by utilizing a primer derived from this sequence together with a primer consisting of telomeric repeats. Together, these findings suggest that this repetitive sequence is subtelomeric on some chromosomes.

The Southern blot data showed a hybridization of a probe containing this sequence not only with human and chimpanzee but also with baboon DNA, indicating a greater conservation of this sequence than previously suggested (7). The Southern blot data also suggest that the occurrence of this fragment in tumor 2-D DNA gels was the result of partial demethylation at *Not* I sites in the repetitive DNA sequence. The difference in methylation at *Not* I sites in the tumor relative to normal tissue is attributable either to demethylation involving a fraction of the repetitive DNA sequences, as may apply to a single chromosome, or to complete demethylation of *Not* I sites in a fraction of tumor cells. The latter possibility is unlikely, since multiple samplings of tumor tissue yielded identical intensities for the repetitive DNA fragment.

The 2-D analysis of genomic digests is a particularly powerful approach for the study of methylation changes based on the use of *Not* I or other methylation-sensitive enzymes. Thus Miwa *et al.* (21) have detected one multicopy fragment in DNA from malignant melanoma, colon cancer, and pancreatic cancer cell lines by 2-D DNA electrophoresis. It was shown that the presence of the multicopy fragment in tumor patterns was due to partial demethylation of a normally methylated genomic repeat sequence of 13 kb (21).

Our findings indicate that only a fraction of repetitive units exhibit demethylation at the *Not* I sites. If cytosine methylation is dependent only on the DNA sequence or is a random process, it would follow that the multiple copies of a repetitive sequence should all be methylated, completely demethylated, or randomly methylated. Since the data indicate that a tandem subset is demethylated, it would appear that factors in addition to the sequence motif, such as chromosomal location, play a role in the methylation process.

An interesting finding from our study and from the study of Miwa *et al.* (21) is the correlation of demethylation of repetitive sequences with malignancy. In both studies, partial demethylation was observed in tumor cells but not in normal cells, including Epstein–Barr virus-transformed lymphoid cell lines. Furthermore, experiments in which normal proliferating lymphoid cells were treated with a methylation inhibitor and subjected to 2-D DNA analysis showed that the methylation of the *Not* I site in the repetitive sequence is not labile. In these experiments, treatment with the methylation inhibitor procainamide did not result in the occurrence in the 2-D pattern of the *Not* I–*Hin*fI fragment in the repetitive sequence (D.T. and B.R., unpublished results). Altered DNA methylation is a common feature in human cancers. A decreased level of overall DNA methylation has been observed in fibrosarcoma, neuroblastoma, and rhabdomyosarcoma (22). In contrast,

Baylin *et al.* (23, 24) observed substantial hypermethylation in specific regions of the human genome in some tumors. It follows that alteration in the methylation status of defined sequences, either hyper- or hypomethylation, may be implicated in malignant transformation.

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