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Genetics

Isolation and characterization of allelic losses and gains in colorectal tumors by arbitrarily primed polymerase chain reaction

(DNA fingerprinting/gene deletion/cancer cytogenetics)

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ABSTRACT **The arbitrarily primed polymerase chain reaction (AP-PCR) [Welsh, J. & McClelland, M. (1990) *Nucleic Acids Res.* 18, 7213–7218] has been used to detect somatic genetic alterations in tumors of the colon and rectum. DNA fingerprints generated by single arbitrary primers were compared between normal and tumor tissue of the same individuals. AP-PCR bands showing decreased and increased intensities in tumor tissue DNA, relative to normal, have been cloned after reamplification with the same arbitrary primer. Standard restriction fragment length polymorphism and Southern blot analyses show that these DNA sequences have undergone allelic losses and gains, respectively, in the tumor cell genome. The deleted sequences have been assigned to the short arm of chromosome 17 by PCR of somatic hamster/human cell hybrids and linkage analysis. These results show the ability of the AP-PCR to detect and isolate, in a single step, DNA sequences representing two of the genetic alterations that underlie the aneuploidy of cancer cells: losses of heterozygosity and chromosomal gains. Altogether, they also show the quantitative nature of the amplification levels obtained *in vitro* by AP-PCR, which thus provides the basis for an alternative molecular approach to cancer cytogenetics.**

The search for genetic alterations that may play a causative role in tumorigenesis has been rewarded by the frequent finding in tumor cells of mutations in protooncogenes (1) and tumor suppressor genes (2). Other genomic DNA sequences are irremediably altered in transformation as a consequence of their linkage to these critical genes. An average of about 20% of the genomic sequences undergo losses of heterozygosity (LOH) in colorectal cancer (3). To a great extent, this is due to the loss of chromosomal sequences that accompanies the inactivation of tumor suppressor genes (4). In addition to allelic losses, increases in chromosomal sequences in the tumor cell genome relative to the normal cell genome underlie the aneuploidy of the cancer cell observed cytogenetically (5).

Differential cloning approaches using genomic DNA have been used to isolate sequences that differ between two otherwise identical genomes, as is the case of sequences deleted during tumorigenesis. These methods use consecutive enriching steps by subtractive annealing of cellular DNA (6). Isolation of DNA sequences based on their amplification also has been described, using differential hybridization and enrichment by in-gel renaturation (7).

DNA fingerprinting is a powerful technique for the comparative analysis of closely related genomes and has shown forensic and medical applications (8). DNA fingerprinting using the highly polymorphic variable number of tandem repeats (VNTRs) (9) also has been applied to the detection of polymorphisms during malignant transformation (10, 11) and to studies of clonality of tumors, both primary and metastatic (12, 13). The polymerase chain reaction (PCR) (14, 15) has been extensively utilized for the detection and characterization of mutations associated with tumor development (16). DNA fingerprinting is one of the techniques that has benefited from the advent of the PCR (17).

We have used a PCR-based DNA fingerprinting technique to study the genetic alterations occurring in tumor cells. The method, called arbitrarily primed PCR or AP-PCR by Welsh and McClelland (18), utilizes amplification with a single arbitrary primer. The first cycles of amplification are performed at a low annealing temperature, which is raised in subsequent cycles. In the initial cycles, the primer hybridizes to many sequences in the total genomic DNA. When the temperature is increased, only the best matches of the initial annealing events are amplified further, generating a number of discrete bands that provide a fingerprint of the cell genome. This approach has been applied for mapping DNA polymorphisms in various prokaryotic and eukaryotic systems (18–20).

We have found that tumor-specific somatic genetic alterations can be readily detected by comparison of the AP-PCR fingerprints from tumor and normal tissue of the same individual. In addition, and in contrast to the VNTR DNA fingerprinting approach, the AP-PCR method permits the direct cloning of the *in vitro* amplified DNA sequences. We describe here the detection, the isolation, and the chromosomal localization of DNA sequences that have frequently undergone allelic losses in colorectal tumors. We also show that the AP-PCR method is useful for detection and isolation of DNA fragments corresponding to chromosomal regions present in multiple copies in the tumor cell genome.

MATERIALS AND METHODS

AP-PCR. Most colorectal tumor samples (7 adenomas and 63 adenocarcinomas, of which 6 were metastatic to the liver) were obtained from the Human Tissue Cooperative Network (University of Alabama, Birmingham). Normal colorectal or liver tissue from the same individuals was analyzed concurrently with the tumor samples. Genomic DNA was prepared as described (21). DNA (20–200 ng) was incubated with 1 unit of Taq DNA polymerase (Perkin–Elmer/Cetus or Stratagene), 125 μM each dNTP, 5 μCi of adenosine 5′-[α-[^{35}S]-thio]triphosphate or 1 μCi of [α-^{32}P]dCTP (New England Nuclear; 1 Ci = 37 GBq), 10 mM Tris·HCl at pH 8.0, 50 mM KCl, 5.0 mM $MgCl_2$, 0.1% gelatin, and an arbitrary primer (0.1 μM) in a volume of 25 μl. The arbitrary primers were KpnX, 5′-CTTGCGCGCATACGCACAAC-3′ (18), and MCG1, 5′-AACCCTCACCCTAACCCCAA-3′. The reactions were carried out in a thermal cycler (Perkin–Elmer) for 5 cycles of low stringency (95°C for 30 or 60 sec, 40 or 50°C for 1 min, and 72°C for 1.5 or 2 min, for primers MCG1 and

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Abbreviations: AP-PCR, arbitrarily primed polymerase chain reaction; LP, length polymorphism; RFLP, restriction fragment length polymorphism; LOH, losses of heterozygosity; nt, nucleotide(s).

KpnX, respectively) and 30 cycles of high stringency (95°C for 15 or 30 sec, 60°C for 15 or 30 sec, and 72°C for 1 min). Two microliters of the PCR product was analyzed in denaturing sequencing gels (ref. 22, pp. 13.45–13.57).

Cloning of Genomic DNA Sequences Amplified *in Vitro* by AP-PCR. The excised bands from the gels were incubated in water at 60°C for 10 min. One microliter of the eluted DNA was reamplified with the same AP-PCR primer as before, with a $MgCl_2$ concentration of 2.5 mM for 30 high-stringency cycles. The PCR product was analyzed in a polyacrylamide gel to confirm its size and purity. The amplified DNA band was isolated from the gel, digested with *Bss*HII (site present in the KPNX primer) or treated with the Klenow fragment of *Escherichia coli* DNA polymerase (for the bands amplified with the MCG1 primer) and cloned in the *Bss*HII or *Sma* I site of the Bluescript II plasmid vector (Stratagene), respectively.

DNA Sequencing, PCR Amplification, and Restriction Fragment Length Polymorphism (RFLP) Analyses. Cloned AP-PCR DNA fragments were sequenced by using a cycling protocol (M.A.P., M. Fernandez-Renart, G. Capella, L. Wilson, and M.P., unpublished work). PCR primers were designed to amplify these sequences directly from genomic DNA (AU3, 5′-GGGGCTAGTCACCCACATTA-3′; AD618, 5′-TGGGGAATGTGACGGTCAAT-3′). PCR was performed as described above, using high-stringency conditions for 35 cycles. Polymorphism studies were performed with radiolabeled PCR products by adding 1 μCi of [α-^{32}P]dCTP to the reaction mix. For length polymorphism (LP) analyses, samples were analyzed in a sequencing gel after heating for 3 min at 95°C. RFLP analysis of DNA samples digested with *Hin*dIII was performed by nondenaturing polyacrylamide gel electrophoresis.

Chromosomal Localization of Cloned Sequences. DNA panels of rodent/human somatic cell hybrids, the PCR-amplifiable panel I from Bios (New Haven, CT) and the National Institute of General Medical Sciences mapping panel II from Coriell Institute for Medical Research (Camden, NJ), were used to determine the chromosomal localization of the cloned DNA sequences. PCR was performed with 50–100 ng of genomic DNA from the different hybrids using specific PCR primers (AU3 and AD618).

Southern Blot Hybridization. Ten micrograms of genomic DNA was digested with *Hin*dIII, *Eco*RI, or *Msp* I and electrophoresed in 0.8% agarose gels. Southern blot analyses were performed as described (21). Prime-It kit (Stratagene), based on the random primer method (23), and PrimeErase Quik columns (Stratagene) were used to label and purify the probes, respectively. In addition to the hybridization of cloned AP-PCR bands, the following chromosome 17 polymorphic probes were used: pYNZ22 (17p13.3) (3), pMCT35.1 (17p13.1-p11.2) (3), and pC63 (17q23-qter) (24).

RESULTS

AP-PCR DNA Fingerprinting of Colorectal Tumors. The results of typical AP-PCR experiments of matched colorectal normal–tumor tissue DNA pairs are shown in Figs. 1 and 2, each using a different arbitrary primer. More than 50 DNA fragments of sizes ranging from <100 to ≈2000 nt were reproducibly amplified with each of the single arbitrary primers. The DNA fingerprints were different, depending on the primer. For a given primer, differences in the band pattern were also apparent. Some of these differences represented polymorphisms in the human population, because they were present in both normal and tumor tissues from only some of these individuals. For instance, a band of ≈900 nt was present only in patient 197 (Fig. 1). Two bands of about 730 nt (A1 and A2, Fig. 1) appeared to represent two alleles from the same polymorphic locus, some individuals with a small A2 allele (patients 135, 116, 78, and 237), others with a large A1 allele (patients 131, 191, 82, 83, and 197), and some others with both alleles (patients 149, 136, 122, and 183). This fingerprinting property of the AP-PCR was useful to ensure that the normal and tumor DNA samples corresponded to the same individuals.

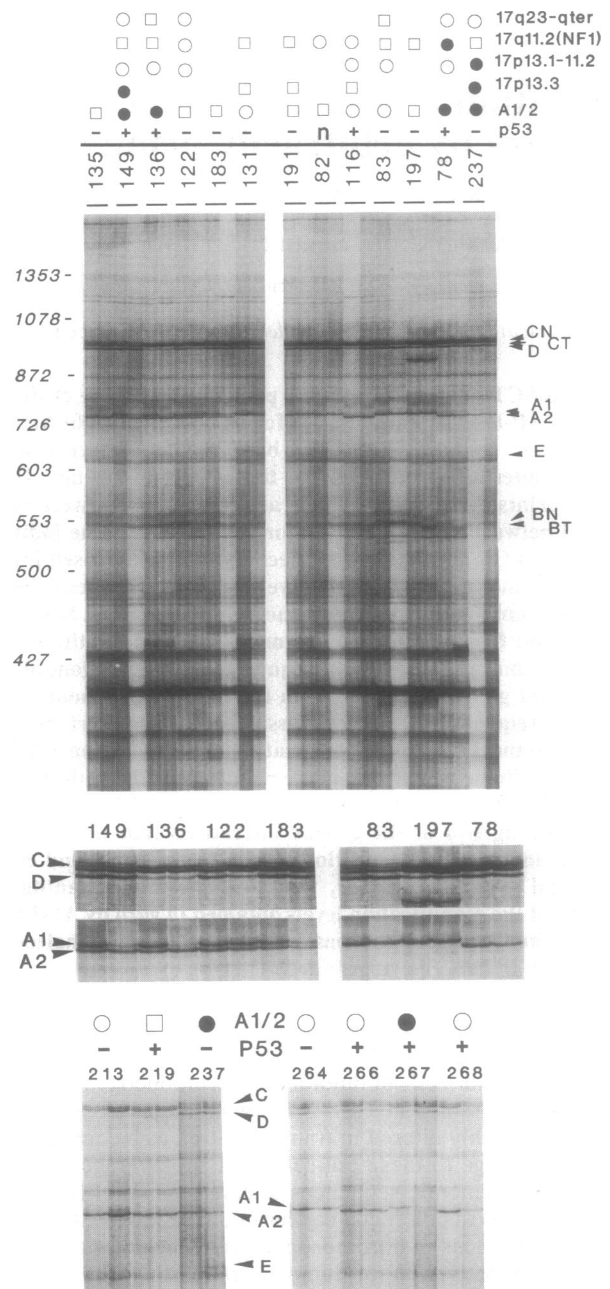

FIG. 1. AP-PCR analysis of colorectal tumors. Autoradiograms of denaturing polyacrylamide gel electrophoresis of ^{35}S-labeled DNA fragments amplified by AP-PCR. Total genomic DNA (50 ng) from normal–tumor tissue pairs of patients indicated at the top was amplified with the KpnX arbitrary primer. In each pair of lanes, the normal tissue DNA is at the left and the tumor DNA is at the right. Size of bands [in nucleotides (nt)] is indicated at the left. Letters at the right (*Top*), left (*Middle*), and middle (*Bottom*) indicate denomination of bands, as used in the text. The summary of the values for the allelic status of the tumors for the A1/A2 and other chromosome 17 sequences is shown at the top. ●, LOH in tumor; □, no LOH; and ○, cases not informative; p53, presence (+) or absence (−) of mutations in the p53 gene; n, not analyzed. A1/A2, summary of allelic status by LP and RFLP (see text).

Somatic Mutations. Other differences in the DNA fingerprint between normal and tumor cells were suggestive of

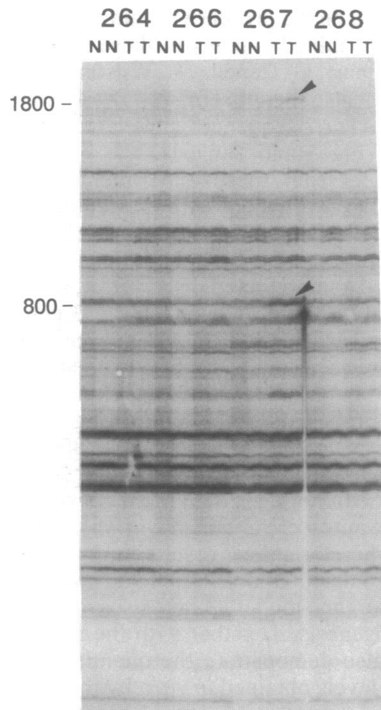

FIG. 2. AP-PCR analysis of colorectal tumors. Autoradiogram of ^{32}P-labeled AP-PCR band pattern of normal (N) and tumor (T) tissue pairs of patients indicated at top, amplified with primer MCG1. Samples were amplified in duplicate, using 50 (left lanes) and 200 (right lanes) ng of genomic DNA. Numbers at left are length in nt.

somatic mutations. For example, tumor 197 showed a band of about 550 nt, apparently of smaller size than that present in the normal tissue (BT and BN, respectively, Fig. 1 *Top*). Patient 197 also exhibited another small change in the mobility of two bands of about 950 nt (CT and CN in Fig. 1 *Top*, both labeled *C* in Fig. 1 *Middle*). We have cloned these altered DNA bands and found that they represent somatic structural alterations in the genome of the cancer cells: specifically, somatic deletions of a few nucleotides that occur in a subset of colorectal tumors, of which tumor 197 is a representative example. The analysis of these genetic alterations will be described elsewhere.

In addition to these qualitative alterations, we also observed decreases and increases in the relative intensities of the bands in tumor versus normal tissue DNA. An example of the first class of changes is band D (case 122, Fig. 1 *Top* and *Middle*). Band A1 also was weaker in tumors 149 and 136, suggesting that the A1 allele had been lost in these tumors. Bands with increased intensity in tumor tissue relative to normal tissue can be seen in Fig. 2 (bands of 1800 and 800 nt indicated by arrowheads). These results suggested that these DNA sequences were present in multiple copies in the cells of tumor 267, a liver metastasis of a colon carcinoma.

We have carried out this study to test the following hypotheses: first, that decreases in the intensity of AP-PCR bands in tumor tissue reflect allelic losses, which could be due to their linkage to suppressor genes; and second, that increased band intensity in tumor tissue DNA reflects the presence of extra copies of these sequences, which could be due to gene amplification or to chromosomal imbalance as a result of the tumor cell aneuploidy.

Isolation of Allelic Losses in Colorectal Tumors. The A1 and A2 DNA bands were cloned and sequenced. Their differences in mobility were due to a deletion/insertion of 6 nt (ATATGT) repeated several times in a stretch of alternating purine-pyrimidines (data not shown). These results confirmed that the A1 and A2 bands represented distinct alleles of a single-copy polymorphic locus. PCR primers were used for the amplification of these sequences from genomic DNA (Fig. 3). A third allele (A3) became evident, which was not observed in the AP-PCR gels (patient 133). A3 contained a duplication of 25 nt relative to A2, also in the variable repeat (data not shown). Another allele (A0) that was not amplified in the AP-PCR experiments was also identified (compare patients 191, 82, 197, and 267 of Fig. 3 with those of Fig. 1 *Top*). The failure to amplify the A0 and A3 alleles by AP-PCR is likely due to sequence differences in the target region for primer annealing. LOH were observed in the tumor tissue of many of the informative cases (267, 121, 133, 134, 136, and 149).

A nucleotide difference found in these DNA sequences generates a *Hin*dIII RFLP that was useful for determination of their heterozygosity by another criterion independent of their LP. The allelic composition of these sequences was estimated by comparing the intensities of cleaved and resistant bands between normal and tumor DNA after *Hin*dIII digestion. While tumors 191, 82, 197, and 219 remained unchanged in their allelic composition, tumors 78, 237, and 267 showed LOH of these sequences (Fig. 4).

Frequency of LOH of A1/A2 Polymorphic DNA Sequences. As predicted by the AP-PCR experiments, the LP and RFLP results show that the polymorphic A1/A2 AP-PCR bands had undergone LOH in about half of the colorectal tumors analyzed (Table 1). By AP-PCR, three of the five patients heterozygous for the A1/A2 alleles showed a reduction of one of the bands in tumor tissue DNA. Of patients amplifying only one of the A1/A2 polymorphic bands, nine tumors showed decreases in the intensity of the band relative to normal tissue (examples are patients 78 of Fig. 1 *Top* and 267 and 268 of Fig. 1 *Bottom*). LOH was confirmed by LP or *Hin*dIII RFLP in all six informative cases.

Among the patients showing no decreases in the intensity of the tumor band relative to the normal by AP-PCR, many also showed no LOH by LP/RFLP (compare patients 135, 122, 183, 191, 82, and 197 of Fig. 1 *Top* and 219 of Fig. 1 *Bottom* with those of Figs. 3 and 4). However, LOH was demonstrated by the LP/RFLP experiments in some cases. Patient 237 is an example of such a false-negative result. While the AP-PCR pattern showed similar intensity in the A2 band from normal and tumor cells (Fig. 1 *Top*), the *Hin*dIII RFLP showed LOH of these sequences in the tumor (Fig. 4). The failure to detect these quantitative differences might be due to a limitation of the AP-PCR technique when the overall levels of *in vitro* amplification are not equivalent (compare the intensities of the A2 bands with the overall level of amplification of tumor versus normal DNA of patient 237 in Fig. 1 *Top*). Another reason may be mitotic recombination or duplication of the remaining chromosome in the tumor cells (25). This appears to be the case in tumor 136 (compare the intensity of the A2 band in tumor versus normal tissue in Fig. 1 *Top* and *Middle*). Losses of the A0 or A3 alleles also would necessarily pass unnoticed.

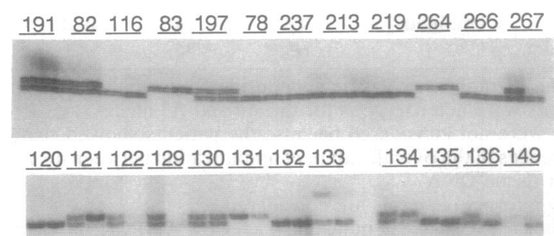

FIG. 3. LP analysis of A1/A2 sequences in colorectal tumors. Autoradiogram of a denaturing polyacrylamide gel of ^{32}P-labeled A1/A2 sequences amplified by PCR with AU3 and AD618 primers, from colorectal normal (left) and tumor (right) tissue pairs from patients indicated at top.

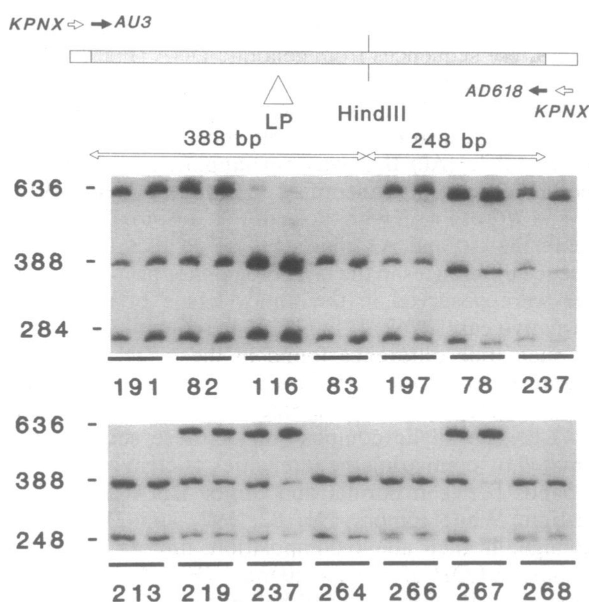

FIG. 4. RFLP analysis of A1/A2 sequences of colorectal tumors. Autoradiogram of a nondenaturing polyacrylamide gel of ^{32}P-labeled DNA fragments amplified by PCR from the normal–tumor tissue pairs indicated at the bottom, digested with *Hin*dIII. bp, Base pairs.

Despite these limitations, AP-PCR was able to determine that deletions of these sequences had occurred in nearly half of the tumors where LOH was demonstrated by LP or RFLP (Table 1). These results demonstrate the ability of AP-PCR to detect and isolate DNA fragments that represent tumor-specific somatic genetic alterations even when these alterations are their own deletion. They also suggest that the A1/A2 polymorphic sequences were linked to one of the tumor suppressor genes involved in colorectal cancer (4).

Chromosomal Localization of A1/A2 Polymorphic DNA Sequences. The chromosomal localization of the A1/A2 sequences was determined by using PCR amplification of panels of rodent/human cell hybrids (see *Materials and Methods*). The results show that these polymorphic sequences were localized in chromosome 17 (data not shown). To map these sequences more precisely, we carried out linkage analysis with other chromosome 17 polymorphic sequences, by standard RFLP using Southern blot hybridization and PCR of DNA sequences with variable-length repeats (data not shown). The results are summarized above

Table 1. LOH of A1/A2 polymorphic DNA sequences

Analysis	No. of cases	LOH	
		No.	%
AP-PCR*	58	12	20.7
LP†	21	11	52.4
RFLP†	39	16	41.0
Combined‡	49	24	49.0
p53§	62	36	58.1

*Of the 70 total cases, 2 were not considered for analysis because the patients were heterozygous for the A0 and A3 alleles, and 10 other cases were not considered because the patients showed significant differences in the overall extent of amplification between normal and tumor tissue DNA.

†Only informative cases are considered.

‡Includes 3 cases that indicated LOH by AP-PCR but were not informative by LP or RFLP.

§Indicates number of cases analyzed and number and percentage of mutated p53 genes. Fourteen of 21 (66.7%) of tumors with p53 gene mutations showed LOH in A1/A2 sequences. In contrast, of 18 tumors negative for p53 gene mutations, only 3 (16.7%) showed LOH in these sequences (P = 0.0017, Fisher exact test).

Fig. 1 *Top*. Data on the presence of mutations in the p53 gene, previously determined in many of these tumors (M.A.P., M. Fernandez-Renart, G. Capella, L. Wilson, and M.P., unpublished results) also was useful for these analyses. Allelic losses of the A1/A2 sequences correlated with mutations in the p53 gene (Fig. 1 and Table 1).

We conclude from these experiments that the A1/A2 polymorphic DNA sequences are located on the short arm of chromosome 17. Therefore, the loss of these sequences in colorectal tumors and their linkage to a tumor suppressor gene, observed and predicted by AP-PCR, are thus explained and confirmed by their linkage to the p53 gene (4).

Detection and Isolation of DNA Sequences Moderately Amplified in Colorectal Tumors. We have cloned the bands of 1800 and 800 nt of tumor 267 (Fig. 2). The cloned DNA fragments were used as probes in Southern blots of genomic DNA from this and other patients. The blots for the 800-nt band (Fig. 5) show that these sequences were about 3- to 4-fold more abundant in the tumor cells than in the normal cells of patient 267. Similar results were obtained with the 1,800-nt band (data not shown). Therefore, these results demonstrate the ability of the AP-PCR to detect and isolate, in a single step, sequences that have undergone moderate increases in their copy number during tumorigenesis. Together with the results of the allelic losses, they also demonstrate the quantitative nature of the amplification levels obtained *in vitro* by the AP-PCR method, therefore validating the working hypotheses stated under *Somatic Mutations*.

DISCUSSION

We have shown that AP-PCR (18–20) is useful for the analysis of the genetic alterations occurring during tumorigenesis. The large number of sequences amplified with the arbitrary primers generates a complex DNA fingerprint that can detect qualitative and quantitative differences between normal and tumor cell genomes, starting with minute amounts of tissue. Because of the arbitrary nature of the priming events, we reasoned that the *in vitro* amplified DNA sequences should be a small but representative sample of the cell genome. Therefore, there should be no bias regarding their chromosomal location. We also reasoned that if these bands were derived from single-copy sequences, and their intensities were proportional to the concentration of the target sequences, then AP-PCR could yield information on the overall chromosomal composition of the cell. By analyzing the genetic alterations occurring during colorectal tumorigenesis, we have confirmed these predictions.

The chromosomal localization of multiple bands can be determined simultaneously by AP-PCR from rodent/human hybrids and comparison with the DNA fingerprints of rodent and human DNA. For instance, in addition to the two bands cloned from tumor 267, another 13 of the major bands amplified with the arbitrary primer of Fig. 2 were assigned to 11 human chromosomes in a single experiment, confirming

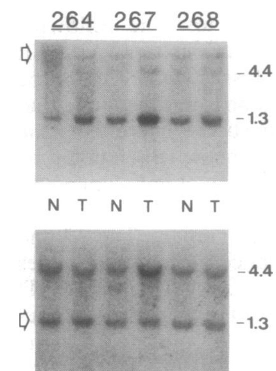

FIG. 5. Southern blot analysis of AP-PCR-cloned DNA sequences from colorectal tumors. Total cellular DNA from normal and tumor tissue pairs from patients indicated at the top digested with *Eco*RI (*Upper*) or *Hin*dIII (*Lower*) and hybridized to ^{32}P-labeled 800-nt DNA fragment from tumor 267 (see Fig. 2). The 17q23-ter probe pC63 was used as an internal control (indicated with an empty arrow). Numbers on right are length in kilobases.

the absence of bias for their chromosomal derivation (unpublished results). All bands that we have analyzed by using different primers were derived from single-copy loci, although some contained repeated sequences. For instance, the B band (Fig. 1 *Top*) was a single-copy sequence although it contained an *Alu* repeat.

The identification of sequences that have undergone allelic losses and gains in tumors proves the quantitative nature of the AP-PCR. However, a technical problem is the occasional occurrence of changes in the intensities of the bands, which appear to be nonreproducible fluctuations in the *in vitro* amplification, probably due to differences in DNA quality. For instance, in some patients (149, 183, 82, and 83), the DNA from tumor tissue was better amplified in its higher molecular weight region than the DNA from normal tissue (Fig. 1 *Top*). In these circumstances, quantitative changes can be artifactual and should not be considered for analysis (Table 1). Nevertheless, when the extent of *in vitro* amplification is about the same for normal and tumor tissue DNA, quantitative differences in band intensity can be accurately recognized.

While LOH can be readily detected by RFLP using Southern blot hybridization (3) or a number of PCR-based techniques (26), none of these methods can formally determine whether the change in the ratio between alleles is due to losses of one or gains of the other. Only the use of other unrelated sequences as internal controls can resolve the issue (25). The AP-PCR method carries these internal controls because of the simultaneous amplification of many independent DNA sequences. Bands close above and below the bands of interest can be used for internal calibration. Moreover, the competitive nature of the amplification events among the different DNA fragments is also an important determinant in its quantitative properties (27).

The AP-PCR allows the direct cloning of sequences representing genomic deletions. In contrast to difference cloning procedures (ref. 6 and references therein), our method does not require the deletions to be homozygous for their detection and isolation. Although the absence of a band is a condition necessary but not sufficient (as shown for the A1/A2 bands), identification and cloning by AP-PCR of homozygous deletions should be also possible, with a number of experiments that must be directly proportional to their size. For instance, a homozygous deletion of 0.1% of the genome should be identifiable by the use of about 20 different arbitrary primers. This calculation is based on an average number of about 50 independent genomic regions that can be viewed with any arbitrary primer (Figs. 1 and 2). As a corollary, the size of the deletions could be estimated by comparative analysis of the total accumulative number of AP-PCR bands necessary for their detection.

The AP-PCR approach is also useful for the detection and isolation of DNA sequences overrepresented in tumors to levels well below the minimum levels required by other available methods (7). The identification and cloning of sequences embodied in amplification units involved in drug resistance and transformation, such as double-minutes or homogeneously stained regions (5), should be also possible with a manageable number of arbitrary primers.

Due to the high level of random genetic damage in the genome of solid tumors (3, 4), the finding that DNA sequences have undergone heterozygous deletions or gains of extra copies in tumor relative to normal tissue does not ensure that these sequences are linked to genes playing an active role in oncogenesis. However, the implication of these sequences in the same type of genetic alterations (i.e., losses or gains), recurring in independent tumors, decreases the probability of isolating DNA fragments corresponding to chromosomal regions randomly altered during tumorigenesis. The assignment to 17p of the A1/A2 polymorphic DNA sequences, initially chosen because of their loss in multiple colorectal tumors, demonstrates the feasibility of the method to identify and isolate DNA sequences corresponding to chromosomal regions relevant to tumorigenesis.

In conclusion, our results show that the AP-PCR permits the cloning, in a single step, of DNA sequences that have undergone the two most common alterations in the cancer cell genome: LOH and gains of extra gene copies. This cannot be achieved by any of the currently available methods, either singly or in combination. The ready determination of the chromosomal localization of these sequences provides a powerful tool for the analysis of the involvement of chromosomal regions in tumorigenesis and the basis for an alternative molecular approach to the cytogenetics of solid tumors.

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