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Genetics

Yeast artificial chromosomes for the molecular analysis of the familial polyposis *APC* gene region

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ABSTRACT **Two yeast artificial chromosomes (YACs) spanning a total distance of 1.1 megabase pairs of DNA around the *MCC* (for *m*utated in *c*olorectal *c*arcinoma) and *APC* (for *a*denomatous *p*olyposis *c*oli) genes at 5q21 have been isolated and characterized. Starting from the *MCC* gene, a strategy was undertaken to identify constitutional submicroscopic deletions in familial adenomatous polyposis patients that might considerably narrow down the position of the *APC* gene. To this end, YACs identified by the *MCC* gene were screened across a chromosome 5-specific cosmid library to provide a source of DNA probes for genomic scanning. The cosmids isolated from these experiments were used to screen a panel of somatic cell hybrids containing chromosome 5 segregated from patients suspected to carry putative interstitial deletions. This screening approach led to the confirmation of a small heterozygous deletion in a polyposis patient that overlaps one of the two isolated YACs. This YAC has been shown to contain the entire *APC* gene, in addition to a significant portion of DNA flanking the 5′ end of the gene, and should therefore prove a valuable resource for functional studies by transfer to colorectal tumor-derived cell lines.**

Familial adenomatous polyposis (FAP) is an autosomal dominant predisposition to colorectal cancer, affecting about 1 in 5000 to 1 in 10,000 individuals in all populations studied (1). The corresponding gene, designated *APC* [for *a*denomatous *p*olyposis *c*oli (2)], was mapped to 5q21-q22 by linkage analysis (3, 4) following a cytogenetic report of a male patient with polyposis and an interstitial deletion on 5q (5). The high incidence of allele loss at 5q21-q22 in carcinomas of sporadic patients (6–9) suggests that *APC* mutations are common in sporadic colorectal adenocarcinomas.

To clone the *APC* gene, our initial studies used two deletions similar to that originally identified by Herrera *et al.* (5) to localize probes to a region around the *APC* gene (10). Subsequently, landmark clones were derived by chromosomal microdissection and microcloning around 5q21-q22 (11, 12). Clones that were localized within the two *APC*-related interstitial deletions were then used to isolate yeast artificial chromosomes (YACs) covering about 4 megabases (ref. 12; G.M.H. and J.R.T.J.W., unpublished results) in order to narrow the search for the *APC* gene among potential candidates.

During this investigation, however, a candidate gene, *MCC* (for *m*utated in *c*olorectal *c*arcinoma), was identified by using a randomly isolated cosmid, L5.71, exhibiting a high frequency of allele loss (13) and which also identified a heterozygous 260-kilobase (kb) deletion in one polyposis family (14). After the discovery of *MCC*, Kinzler *et al.* (15) and Groden *et al.* (16) isolated the *APC* gene itself. In this paper we describe our approach to identifying the *APC* gene, which has led to the isolation of two overlapping YACs containing the *MCC* gene, one of which also includes the complete *APC* gene.

MATERIALS AND METHODS

YAC Methods. The YAC library used in these studies (17) contains approximately two genome equivalents with an average insert size of 600 kb. The library was screened by radiolabeled probe hybridization (11, 12) onto nylon membranes on which YACs were spotted in high-density arrays (18). Potentially positive YAC clones were streaked out on selective media plates lacking uracil, and "micro-blocks" were made from 5 to 10 single colonies as described (19). One-half of such a micro-block was electrophoresed in 1% agarose gels on a Bio-Rad CHEF-DR II apparatus at 5 V/cm for 20 hr with a switching time of 20–84 sec. DNA was transferred to Hybond N^+ (Amersham), and blots were hybridized with appropriate probes to identify true positives. Large-scale agarose block preparations were then made from these clones by standard methods (20, 21). *In situ* fluorescence hybridization of total yeast DNA to human metaphase spreads was carried out by using a modification of the method described by Williams *et al.* (22). Physical mapping of YACs was carried out by partial and total (single and double) digestions using the following restriction endonucleases: *Mlu* I, *Sac* II, *Bss*HII, and *Nru* I (New England Biolabs). Pulsed-field gel electrophoresis resolution conditions were determined by a program supplied by Bio-Rad. Pulsed-field gel electrophoresis blots were initially hybridized with pBR322-derived probes specific for the "right" (defined as that containing the *URA3* gene) and "left" (*TRP1* gene) pYAC4 vector arms. The filters were stripped in boiling 0.5% SDS and subsequently probed with cosmid fragments or gene-specific probes described in the text and figure legends. The left arm-specific insert terminus of YAC A1010 was isolated by *Alu*-vector PCR (23).

Direct Hybridization of YACs on Cosmid Libraries. Agarose blocks from each yeast were electrophoresed in 1% low melting point agarose gels as described above. Appropriate sections of the gel containing the YACs were excised and equilibrated in 10 mM Tris·HCl/1 mM EDTA, pH 8.0 (TE buffer) with three 10-min washes at room temperature. An equal volume of TE buffer was added to the agarose slices; they were heated to 65°C for 5 min and then cooled to 37°C for 10 min. Fifty units of agarase (CalBio) were added, and the samples were left at 37°C for a further 2 hr. After

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Abbreviations: YAC, yeast artificial chromosome; FAP, familial adenomatous polyposis.
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phenol/chloroform extractions, the DNA was precipitated with ethanol using 30 μg of glycogen as a DNA carrier. Approximately 200 ng of purified DNA was labeled by the random priming method using 50 μCi (1 Ci = 37 GBq) of [α-^{32}P]dCTP. Human repetitive sequences were quenched by preannealing the probes with sonicated and sheared human placental DNA (0.7 mg/ml) for 3 hr at 65°C. In addition, human placental DNA was added to a final concentration of 0.015 mg/ml during prehybridization. YAC probes were hybridized to a chromosome 5-specific cosmid library (constructed in vector sCOS-1; provided by L. Deaven, Los Alamos, NM) spotted in high-density gridded arrays and containing approximately two genome equivalents of chromosome 5. Cosmid filters were washed to a final stringency of 0.2× standard saline citrate/0.1% SDS and exposed to Kodak XAR-5 autoradiographic film for 1–3 days. Positive cosmids were picked, and DNA was prepared by standard methods (24). Whole cosmid DNA was labeled by using 20 μCi of [α-^{32}P]dCTP. Hybridizations were carried out essentially as described above.

Somatic Cell Hybrids. Rodent–human hybrids independently segregating both copies of chromosome 5 from two polyposis patients, J.T. (25) and P.S. (26), were generated as described (10). The two somatic cell hybrids, HHW1155 and HHW1159, segregating both copies of chromosome 5 from polyposis patient 3214 were kindly provided by J. J. Wasmuth (see ref. 14).

Probes. cDNA probes were kindly provided by Kenneth Kinzler (The Johns Hopkins University), Bert Vogelstein (The Johns Hopkins University), and Yusuke Nakamura (Department of Biochemistry, Cancer Institute, Tokyo 170, Japan). SW15 and MCC40cI represent nucleotides 133–1918 and 1634–3969 of the *MCC* gene, respectively. TB2 is a cDNA probe containing the entire coding region of the TB2 gene. cDNAs FB9A and FB54D contain nucleotides -22 to 6452 and 6640 to 8954 of the *APC* gene.

RESULTS

Identification and Characterization of YACs Containing the *MCC* Gene. To expand the genomic region around the *MCC* gene, cDNA probes SW15 and MCC40cI (see *Materials and Methods*) were used to screen both the collection of YACs previously localized to 5q21-q22 and the Imperial Cancer Research Fund YAC libraries. Three YAC clones were identified: ICRFy900B0624 (620 kb), ICRFy900B0879 (1200 kb), and ICRFy900A1010 (600 kb), one of which (B0624) had already been identified by microclone mc575 (12). The yeast strain B0624 contains two unrelated YACs, one of which, by *in situ* fluorescence hybridization analysis, maps entirely to chromosome 5 (YAC 624-575) and a second, which maps to a D-group chromosome (YAC 624-D). Similar experiments suggested that YAC B0879 was chimeric with fragments mapping to 5q21-q22 and chromosome 8p, but that YAC A1010 contained DNA entirely from 5q21-q22.

Physical mapping of the 624-575 and A1010 YACs showed them to be overlapping, covering a total distance of ≈1100 kb, with the *MCC* gene centered in the region of overlap and flanked by ≈500 kb of DNA in both directions. YAC A1010 appears to contain the entire coding region of the *MCC* gene, by comparison of its *MCC* cDNA hybridization pattern with that in human DNA (data not shown).

Isolation of Cosmids by YAC Hybridization. YACs A1010 and B0624 were used as probes to screen directly a chromosome 5-specific cosmid library spotted in high-density arrays as described in ref. 18, with the results shown in Table 1. In the initial screens, both YACs identified the same subset of seven cosmids, which represent the overlap between them. This number (25–28% of the total) is consistent with the extent of overlap determined by physical mapping. Of these seven cosmids, two were also identified by a cDNA probe, SW15, representing sequences from the 5′ end of the *MCC* gene. A further eight cosmids from the A1010 YAC were identified by another cDNA probe, MCC40cI, from the 3′ end of the *MCC* gene (see *Materials and Methods*). These results are consistent with the *MCC* Southern blot hybridization results.

Table 1. Hybridization of YACs on chromosome 5-specific cosmid libraries

YAC probe	No. of cosmids	No. common to both YACs	No. of cosmids positive for *MCC*
A1010	29*	7	6
	14†	ND‡	6
B0624	25	7	2

*Derived from screening 9216 clones (1–9216).
†Derived from screening an additional 9216 clones (9216–18,432).
‡Not determined. The B0624 YAC was not screened on this section of the library.

Forty-four of the cosmids from both YAC screens, excluding the overlapping and *MCC*-positive clones, were used for further mapping studies with a panel of somatic cell hybrids containing the segregated chromosomes 5 from FAP patients as follows: (*i*) hybrids HW1155 and HHW1159 from patient 3214 who, at the time of these studies, was thought to have a heterozygous interstitial deletion of about 260 kb on the basis of pulsed-field gel analysis (14); and (*ii*) hybrids JT/Ts-1 and JT/Ts-3 from patient J.T., a Japanese male who exhibited polyposis and mental retardation (25). The association of mental impairment and FAP, seen previously in three Caucasoid FAP patients (5, 10, 27), suggested to us that this patient might also have a submicroscopic deletion of chromosome 5. (*iii*) A somatic cell hybrid, SD/Ts-1, derived from patient P.S. (26), containing a single copy of a chromosome 5 with a deletion around region q22-q23, known to include both YACs (data not shown) was also included to assess each cosmid (12).

Identification of an *APC*-Related Deletion. Initial mapping studies with the cosmids identified from the library filters by the first YAC-probe screens (Table 1) showed that a number did not map within the deletion present in hybrid SD/Ts-1 (Table 2) and were therefore not likely to represent sequences present in the YAC inserts. The reason for this is unknown but may be cross-reaction with repetitive sequences within the YAC. In subsequent hybridization experiments using YAC A1010 (Table 1), all of the cosmids that were identified were shown to map into the SD/Ts-1-containing deletion, as expected for optimized hybridization conditions (Table 2). None of these cosmids showed aberrant hybridization patterns on either hybrid JT/Ts-1 or JT/Ts-3, raising the question as to whether patient J.T. really did carry a microdeletion around the *APC* gene.

Five cosmids identified by the A1010 YAC probe showed hybridization patterns clearly suggesting the presence of a deletion in the homolog of chromosome 5 from patient 3214, which segregated in the HHW1155 hybrid. Ym75 sequences appeared to be entirely absent in this hybrid cell line, whereas

Table 2. Direct screening of YAC-identified cosmids on hybrid panels

YAC	No. of cosmids screened	No. deleted in hybrid SD/Ts-1	No. deleted in hybrid HHW1155
A1010	21*	8 (40%)	2
	8†	8 (100%)	4
B0614	15	9 (60%)	0

*From the first cosmid library screen.
†From the second library screen.

the other four cosmids, ym8, ym21, ym64, and ym72, hybridized to altered sized bands in addition to showing the absence of relevant restriction fragments in HHW1155. These results initially suggested that one or both of the breakpoints of the 3214 deletion had been recognized. Subfragments from cosmids ym8 and ym72 were found to be entirely absent in HHW1155, confirming the presence of a deletion overlapping with YAC A1010. Fig. 1 demonstrates the recognition of this deletion by whole cosmid and cosmid subfragment hybridizations.

Since cDNA probes from the *MCC* gene were not visibly rearranged in the HHW1155 cell line, it was reasoned that the deletion must map distant to the 3′ end of the *MCC* gene and toward the left pYAC4 arm of the YAC (see Fig. 2). Comparison of hybridization patterns between the set of four "partially" deleted cosmids initially suggested that three were overlapping (ym21, ym64, and ym72), in addition to recognizing a consistent altered sized band of 5.9 kb in hybrid HHW1155. Cosmid ym8 was subsequently found to overlap with cosmid ym21 by restriction digestion analysis. However, cosmid ym8 revealed an altered sized band of 5.1 kb, different from that of the other cosmids, and thus inconsistent with these four cosmids overlapping a single breakpoint of a simple deletion. Nonetheless, these cosmids, and in particular cosmid ym75, were thought to represent ideal candidates for containing sequences of the *APC* gene.

However, at this time, an additional three genes, TB2 (or DP.1), SRP19, and *APC* (or DP2.5), were identified within overlapping interstitial deletions from two FAP patients, including the chromosome 5 deletion from patient 3214 (14, 15). Cosegregation of mutations in one of these genes, *APC* (or DP2.5), with polyposis (16, 28) and the demonstration of germ-line transmission of a new mutation (16) confirmed this candidate gene as *APC*.

The *APC* Gene Is Contained on YAC A1010. cDNA clones from the *APC* gene (FB9A and FB54D) and from the TB2 gene (see *Materials and Methods*) were used to screen digests of the YAC A1010 and show that both genes were present on this YAC, in addition to about 160 kb of DNA flanking the 5′ end of the *APC* gene (Fig. 2). The left insert terminus of this YAC was isolated by *Alu*-vector PCR (23) and mapped to chromosome 5, which shows that it probably contains most of the relevant control elements at the 5′ end of the gene.

A number of *Nru* I and *Mlu* I sites in the physical map, particularly at the 5′ end of the TB2 gene and surrounding the *APC* gene, agree with those described by Joslyn *et al.* (14) and confirm that the 3214 deletion extends distally from the *MCC* gene to encompass most of the *APC* gene (marked in circles in Fig. 2). The central *Bss*HII and *Mlu* I sites that separate the *APC* and *MCC* gene regions are also in agreement with a genomic physical map constructed by one of us (J.R.T.J.W., unpublished results). It has in addition been possible to confirm the relative transcriptional orientation of the *MCC* and *APC* genes as being toward each other, by mapping 5′ and 3′ derived cDNA probes on subdefined restriction fragments of the YAC. The 5′ end of the TB2 gene is associated with a CpG island, but neither the *MCC* nor *APC* genes are.

APC and TB2 cDNA probes were also used to screen restriction digests of the five cosmids that revealed the 3214 deletion. Cosmids ym72 and ym75 were shown to contain sequences from the 3′ and 5′ ends of the *APC* gene, respectively, while cosmids ym8, ym21, and ym64 were shown to contain portions of the TB2 gene. These cosmids have been placed in their most likely positions on the YAC-based physical map shown in Fig. 2. The combined results of all of these analyses strongly suggest that none of these cosmids (excluding ym75) should have revealed altered sized restriction fragments in hybrid HHW1155, because they are all

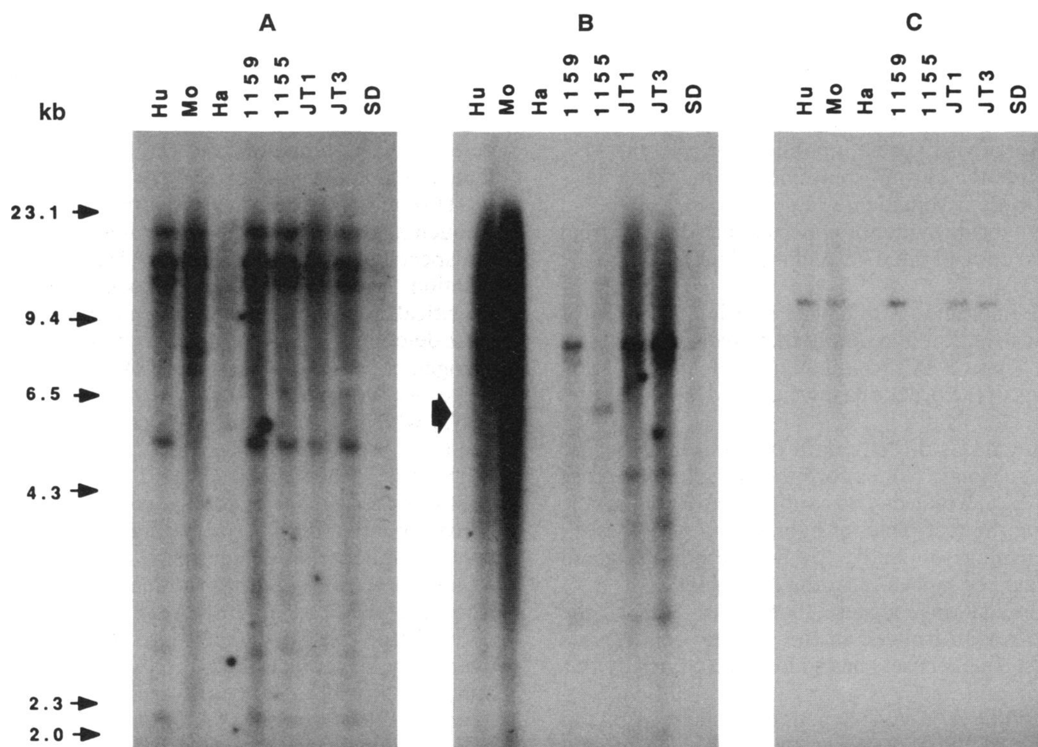

FIG. 1. Identification of an interstitial deletion in patient 3214. Hybridization on somatic cell hybrid panels using A1010-identified cosmids ym2 (not deleted; *A*), ym21 (deleted; *B*), and a subfragment derived from cosmid ym72 (deleted; *C*) is shown. The identities of genomic DNAs are indicated above the figure. Hu, human; Mo, mouse; Ha, hamster; 1159, HHW1159; 1155, HHW1155; JT1, JT/Ts-1; JT3, JT/Ts-3; SD, SD/Ts-1. The sizes of fragments calculated from coelectrophoresis of bacteriophage λ DNA digested with *Hin*dIII are indicated to the left of the figure. The 5.9-kb altered sized fragment revealed by cosmid ym21 is indicated by the arrow to the left of *B*.

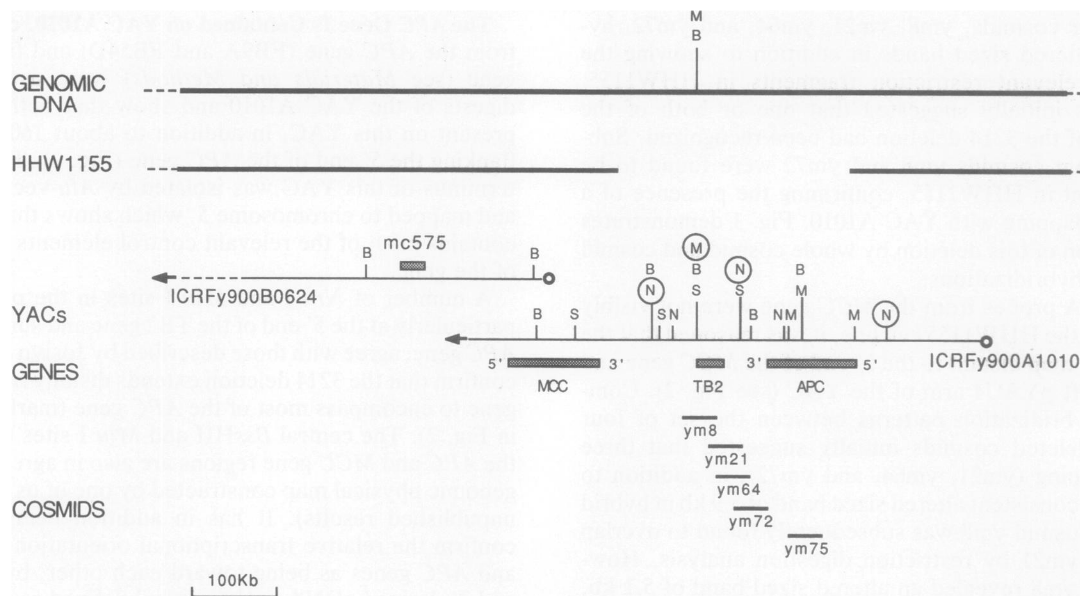

FIG. 2. A detailed YAC-based physical map of the *APC* gene region. Shown here is a YAC-based physical map of the genomic region around genes *APC*, TB2, and *MCC*. Chromosome 5 retained in hybrid HHW1155 is shown as a broken line. The position and extent of the deletion are based on the map reported by Joslyn *et al.* (14) and confirmed here by a combination of Southern blot hybridization analyses and YAC-based physical mapping using cDNAs from the *MCC*, TB2, and *APC* genes. The dashed line in YAC B0624 indicates that this YAC extends in the direction of the arrow (total size of 620 kb). Only a partial map of this YAC is shown. The left (*TRP1* gene) and right (*URA3* gene) pYAC4 vector arms of the YACs are indicated by the circles and arrows, respectively. Restriction sites are depicted above the lines representing genomic and YAC DNA: B, *Bss*HII; M, *Mlu* I; N, *Nru* I; S, *Sac* II. The restriction sites outlined by circles represent the equivalent sites described by Joslyn *et al.* (14). The positions of the cosmids (average insert size = 40 kb) are based on hybridization analyses with *APC* and TB2 cDNAs (see text). The positions and genomic distances of the *MCC*, *APC*, and TB2 genes are inferred from physical mapping experiments on restriction digests of YAC A1010 only.

predicted to lie entirely within the deletion. The explanation for the anomalous bands is not yet clear, but it may be that the deletion in 3214 did not arise as a single simple event.

DISCUSSION

We have demonstrated the success of the following hierarchical approach toward the isolation of the *APC* gene:

(*i*) Identify a set of visible deletions encompassing the *APC* gene and segregate the chromosomes containing these deletions in human–rodent somatic cell hybrids (10).

(*ii*) Use these hybrids to identify microclones derived from the physical dissection of the *APC* region, which map into the deletions (11, 12).

(*iii*) Use these microclones to isolate a series of YACs for the eventual construction of a contig that spans the minimal deletion region. These YACs would then enable the detection of microdeletions of the order of a few hundred kilobase pairs or less.

(*iv*) Screen the YACs directly onto chromosome-specific cosmid libraries to isolate clones corresponding to sequences present in the YACs, which can be confirmed to lie within the deletion by using the test panel of hybrids.

(*v*) Use the cosmids to identify, by Southern blotting and other approaches, sequences from the *APC* gene.

Preliminary indications suggest that about half of the minimal deleted region isolated in the somatic cell hybrids (probably at least 8 megabases) has so far been cloned by this approach.

The isolation of the *APC* gene depended critically, first, on the identification of the rare larger deletions such as that described by Herrera *et al.* (5) and, then, on the 260-kb deletion identified in patient 3241 by cosmid L5.71 (14). The combination of developmental abnormalities and mental retardation with polyposis associated with deletions (5) is probably dependent on gene dosage for one or more critical genes within the deleted region. It may, however, also depend on the existence of one or more recessive mutations within the deleted region present on the matching normal chromosome. In that case, apparently similar deletions could give rise to widely differing phenotypic effects.

In our search for smaller deletions using pulsed-field gel electrophoresis, we have found no examples in affected individuals from some 50 polyposis families and in 20–30 colorectal carcinoma-derived cell lines. Two such deletions, including that from patient 3214, were identified by Joslyn *et al.* (14) among ≈40 polyposis patients. This suggests a frequency of, at most, 1% for such smaller deletions among the spectrum of *APC* mutations. Clearly, screening for such deletions using whole YACs, or cosmids derived from them, is critical for gene localization. In our study, the heterozygous deletion from patient 3214 would eventually have been recognized using this approach. Once we knew of its existence, we were able to identify cosmids, such as ym72 and ym75, which we now know to contain portions of the *APC* gene.

The task of identifying the *APC* gene in the absence of the 3214 or other similar deletions would have been very much harder. We found only a very small proportion of colorectal carcinoma-derived cell lines showing detectable changes on straightforward Southern blot analysis using cDNA probes from the *APC* gene. However, a screen using cosmids derived from YACs mapped into the larger deletion might eventually have identified the *APC* gene. An alternative approach would have been to seek first for the functionally expressed genes on the YACs themselves and then to use only these in such a screen. Since the YAC contig we have defined was, in part, identified by one of our microclones, a screen along these lines with the YACs we had isolated presumably would have, in time, been successful. However, the relative effort involved clearly emphasizes the importance of screening for sufficiently small deletions within

which there are only a few potential candidate genes to be screened for mutations segregating with the polyposis phenotype.

What would have been the situation if we had had access to a complete physical and genetic map of all functional genes, the eventual goal of the Human Genome Project? Five megabases is approximately 1/600th of the total genome, and thus could contain between 100 and 200 protein-coding sequences. Taking into account functional clustering, this may represent no more than 20–40 functionally different types of gene clusters. With luck, a number of these could plausibly be eliminated as potential *APC* candidates, leaving a relatively limited number of candidate genes to be tested for potential mutations in FAP patients.

Microcell-mediated transfer of chromosome 5 to colorectal tumor-derived cell lines has been suggested to show suppression of tumorigenicity in nude mice with an associated alteration in cellular morphology (29, 30). The A1010 YAC isolated in our investigation provides a further valuable tool for the study of the function of the genes in this region. Introduction of the YAC, or fragments of it containing either the *MCC* or *APC* genes alone, into appropriate colorectal cancer-derived cell lines of known mutational status should allow a more precise analysis of the function of the *APC* gene.

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