A CA-Repeat Polymorphism Close to the Adenomatous Polyposis Coli (APC) Gene Offers Improved Diagnostic Testing for Familial APC

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Summary
Presymptomatic genetic testing for the presence of a mutant allele causing familial adenomatous polyposis coli (APC) has been difficult to perform effectively in the past because DNA markers surrounding the APC gene on chromosome 5q have not been very informative. We report results of genetic linkage studies on both research families and clinical families by using DSS346, a highly polymorphic dinucleotide (CA)-repeat locus 30–70 kb from the APC gene. Linkage analysis with this marker in a large APC pedigree showed an increase of at least 9.0 LOD units, in likelihood of linkage of the disease-causing allele to the APC locus, when compared with the highest LOD score attained with any other closely linked marker. When the first 14 APC families that requested genotypic analysis by the DNA Diagnostic Laboratory at the University of Utah were tested with DSS346, 20 of the 31 at-risk individuals were identified as either carriers or noncarriers of an APC-predisposing allele. We see this marker as an important tool for research studies and for the presymptomatic diagnosis of APC.

Introduction
A rare inherited predisposition to colon cancer, adenomatous polyposis coli (APC), is characterized by hundreds to thousands of adenomatous colonic polyps that usually develop early in adulthood (Burt and Samowitz 1988). APC is inherited in an autosomal dominant manner and includes both familial adenomatous polyposis (FAP) and Gardner syndrome (GS). GS differs primarily from FAP by the presence of certain benign bone and soft-tissue tumors (Burt 1991). A few years ago, the genetic defect in APC was mapped to human chromosome 5q21-22 (Bodmer et al. 1987; Leppert et al. 1987). Subsequently, the gene responsible for the disease was identified, and inactivating mutations were demonstrated in the coding sequence of APC patients (Groden et al. 1991; Joslyn et al. 1991; Kinzler et al. 1991a; Nishisho et al. 1991; Miyoshi et al. 1992).

Because most of the germ-line APC mutations characterized thus far are different from each other and because more than 50 have been described to date (Groden et al. 1991, 1993; Nishisho et al. 1991; Fodde et al. 1992; Miyoshi et al. 1992; Olschwang et al. 1993; Varesco et al. 1993), one must usually scan the entire open reading frame of this large (8.5-kb) gene to identify the specific mutation in any particular patient. Hence, screening for individual mutations in the APC gene is not yet a practical approach for identifying carriers of disease-causing alleles, and presymptomatic diagnosis must (for the foreseeable future) rely on demonstration of coinheritance of the disease allele with one or more nearby genetic markers. Until recently, most of the DNA markers available for the presymptomatic diagnosis of familial APC were two-allele RFLPs located 1–10 cM from the APC gene. Often, these markers, including those that are relatively close (approximately 1 cM) to the APC gene, are not sufficiently informative for genetic diagnosis of individuals, in nuclear two-generation families, who carry a high risk for developing APC at a relatively early age.

Previously, an effort was undertaken to obtain a highly polymorphic, more closely located marker to use...
for research purposes and for presymptomatic diagnosis of familial APC. A DNA sequence containing multiple CA repeats, lying only 30–70 kb from the APC gene (Joslyn et al. 1991), revealed at least 13 different alleles in the general population; the observed heterozygosity was approximately 83% in 162 unrelated individuals (Spirio et al. 1991). Few recombinational events would be expected to occur between this marker, DSS346, and the APC gene.

In the study reported here, Mendelian inheritance of polymorphisms at this locus was demonstrated in three Centre d’Etude du Polymorphisme Humain (CEPH) reference families. Next, DNA samples from a large polymorphism kindred were used to establish genetic linkage between the CA-repeat marker and the APC locus; the mutation in this family had been mapped earlier to the APC locus by linkage analysis (Leppert et al. 1987; Nakamura et al. 1988). Finally, the CA-repeat marker was tested in 14 families that had been examined previously with several APC markers, at the University of Utah DNA Diagnostic Laboratory, in an attempt to obtain more accurate diagnostic information about the carrier status of individuals, in these families, who are at risk for APC.

Subjects, Material, and Methods

Families

DNA from three, three-generation families from among the reference panel maintained by the CEPH in Paris was chosen to establish Mendelian inheritance of the polymorphisms at this locus. Kindred 1441 is a large APC family that showed linkage of the APC phenotype to 5q in the original mapping studies (Leppert et al. 1987) and that was subsequently utilized in more precise mapping studies (Nakamura et al. 1988). Twenty-five of the 60 individuals sampled from kindred 1441 have been diagnosed with the disorder. The small families tested here were the first 14 families to request APC genotypic analysis by the DNA Diagnostic Laboratory at the University of Utah. Each family contained at least two individuals in whom APC had previously been diagnosed by clinical criteria. Patients were examined at the University of Utah in the Gastroenterology Division or at gastroenterology centers elsewhere.

DNA Isolation, Southern Transfer, and Hybridization

Total genomic DNA was purified from peripheral lymphocytes by phenol/chloroform extraction followed by ethanol precipitation. For RFLP markers, restriction-endonuclease digestions were performed on 5-μg aliquots of each DNA by using MspI, PstI, or TagI. The samples were digested with 2 units of enzyme/μg of DNA, under conditions specified by the manufacturer (Molecular Biology Resources, Milwaukee). The DNA fragments were electrophoresed on 0.8%–1.0% agarose gels and were transferred to Hybond N+ membrane (Amersham, Arlington Heights, IL) in a 0.4 N NaOH solution. After an overnight prehybridization in 7% polyethylene glycol, 10% SDS, 50 mM phosphate buffer, and 200 μg of total human placent DNA/ml, the membranes were transferred to fresh hybridization solution, and the radiolabeled probe was added. To eliminate nonspecific binding of a DNA probe to the genomic DNA samples being tested, it was necessary to include total human DNA, instead of salmon-sperm DNA, in both hybridization solutions. The DNA probes were labeled with [α-32P]dCTP by using the Prime-It™ random primer kit (Stratagene, San Diego), and hybridization was performed for approximately 12 h. Filters were washed twice in 2 × SSC and 0.1% SDS, then once in 0.1 × SSC and 0.1% SDS, each time for 15 min at room temperature. If necessary, a final wash was performed for 2–15 min at 65°C in 0.1 × SSC and 0.1% SDS. The membranes were exposed to X-ray film for 12–48 h at −80°C and were analyzed for RFLPs.

PCR Followed by Formamide Denaturing Acrylamide-Gel Electrophoresis

PCR was used to amplify the DSS346 locus from total genomic DNA. The PCR mixtures consisted of the following reagents: 200 ng of DNA, 0.5 μM of each primer (LNS-CAI and LNS-CAII) (Spirio et al. 1991), 0.01 μM of [γ-32P]ATP end-labeled primer (LNS-CAII); 200 μM of each deoxynucleoside triphosphate, 10 mM Tris-HCl (pH 8.4), 40 mM NaCl, 2.0 mM MgCl2, 1.25 U of Taq DNA polymerase, and 0.25 mM spermidine, in a volume of 50 μl. The end-labeled primer (LNS-CAII) was prepared as follows: 0.1 μM of LNS-CAII, 50 mM Tris-HCl, 10 mM MgCl2, 5.0 mM DTT, 8.4 U of polynucleotide kinase, and 6.0 μl of [γ-32P]ATP (3,000 Ci/ml), in a total volume of 10 μl. This mixture was incubated at 37°C for 30 min and then was heated to 90°C for 2 min to inactivate the T4 polynucleotide kinase, before the PCR was performed. The PCR procedure was as follows: (1) 1 cycle at 94°C for 5 min; (2) 30 cycles, each at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and (3) cooling to room temperature. After PCR, 25 μl of stop dye was added to each sample; the stop dye consisted of 98% formamide, 0.05% bro-
mophenol blue, 0.05% xylene cyanol, and 20 mM EDTA (pH 8.0). Three microliters of each sample were then loaded onto a 7% acrylamide gel that contained 5.6 M urea, 32% formamide, 90 mM Tris-borate (pH 7.5), and 2 mM EDTA (pH 8.0). The preelectrophoresis of the gels (for about 1 h, prior to loading) and electrophoresis were performed at room temperature, with constant power (90 W/gel) in a 90 mM Tris-borate (pH 7.5) and 2 mM EDTA (pH 7.0) running buffer. Loaded gels were electrophoresed for 2-3 h. The wet gels were placed directly onto filter paper (Whatman International, Maidstone, England) and were exposed to X-ray film for 30 min to overnight, at −80°C, and autoradiograms were analyzed for genotypes of the CA-repeat alleles.

**DNA Markers, Genotyping, and Linkage Analysis**

Four of the RFLPs tested here—L5.79, L5.71, EF5.44, and EW5.55—were reported relatively recently (Human Genome Mapping 10.5, Oxford Conference 1990; Petersen et al. 1990; Joslyn et al. 1991; Kinzler et al. 1991b). The physical locations of probes L5.79 and L5.71 were described by Joslyn et al. (1991). The remaining RFLP markers—KK5.33, YN5.64, YN5.48, MC5.61, and EW5.5—were described elsewhere (Nakamura et al. 1988). A genetic map of all the markers (proximal to distal on chromosome 5q) is indicated by the listed order in table 1.

**Table 1**

<table>
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<tr>
<th>Probe*</th>
<th>Enzyme</th>
<th>Recombination with APC (θ)</th>
<th>Allele Sizes (kb)</th>
<th>Constant Bands (kb)</th>
<th>Heterozygosity</th>
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<td>cKK5.33 (DSS58)</td>
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<td>Psfl</td>
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<td>5.0/4.3/4.0</td>
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<tr>
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<td>TaqI</td>
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<td>12.0</td>
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<tr>
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<td>......</td>
<td>Psfl</td>
<td>.045*</td>
<td>5.2/5.5</td>
<td>7.8/4.9/4.0</td>
</tr>
<tr>
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<td>......</td>
<td>TaqI</td>
<td>.045*</td>
<td>8.0/6.5</td>
<td>5.8/5.3/3.0</td>
</tr>
<tr>
<td>pl5.79-2 (DSS138)</td>
<td>......</td>
<td>MspI</td>
<td>&lt;.01 e</td>
<td>1.8/1.5</td>
<td>1.2/1.0</td>
</tr>
<tr>
<td>pl5.79-2 (DSS138)</td>
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<td>Psfl</td>
<td>&lt;.01 e</td>
<td>9.0/7.8</td>
<td>4.7</td>
</tr>
<tr>
<td>pl5.71-3 (DSS141)</td>
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<td>MspI</td>
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<td>4.7/4.5</td>
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</tr>
<tr>
<td>LNS-CA (DSS346)</td>
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<td>...</td>
<td>.096–.122</td>
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<td>MspI</td>
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<td>TaqI</td>
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<td>MspI</td>
<td>.094*</td>
<td>5.9/4.7</td>
<td>...</td>
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<tr>
<td>cEW5.5 (DSS133)</td>
<td>......</td>
<td>MspI</td>
<td>.094*</td>
<td>6.5/4.8</td>
<td>...</td>
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</table>

* Order of probes is from centromere to telomere.
* Authors’ unpublished data.

DSS346 was ascertained from a cDNA clone (GJ31) isolated from a fetal brain CDNA library that contained sequences from the DP1 gene (Spirio et al. 1991). DP1 spans approximately 30 kb of genomic DNA; its open reading frame contains 631 nucleotides and a 2.5-kb 3’ untranslated region. The DSS346 CA repeat is within the 3’ untranslated portion of DP1. The 3’ end of APC is on the same 120-kb _MluI_ restriction endonuclease fragment as _DP1_; therefore, DSS346 is approximately 30–70 kb downstream of the APC gene (Joslyn et al. 1991). All of our genotypic data were gathered in a blind fashion, so that the person assigning the alleles would not know the affection status and pedigree position of any family member being tested.

The _MLINK_ subroutine of the computer program _LINKAGE_ was used for pairwise linkage analysis between the APC locus and the DSS346 marker (Lathrop et al. 1985). The analysis was performed under assumptions both of an autosomal dominant mode of inheritance and an allele frequency of 1/1,000 for the disease
gene in the population. Penetrances of both .5 and .7 were tested. The parameters used above are consistent with those used elsewhere for linkage analyses in APC and GS families (Leppert et al. 1987; Nakamura et al. 1988).

Results

Mendelian inheritance of the DSS346 CA-repeat marker was demonstrated in the three CEPH reference families sampled. One of these pedigrees, kindred 1347, is depicted in figure 1. Both parents of the 10 offspring are heterozygous for DSS346, and their four alleles are evenly distributed among the 10 children.

Two-point linkage analysis between the DSS346 CA-repeat marker and the disease locus was performed for kindred 1441. Maximum LOD scores of 10.21 and 11.24 were calculated at recombination fraction (θ) = .001, when the assumed penetrances of the disease allele were .5 and .7, respectively. The highest LOD score previously obtained for this family was 1.67, with marker YN5.48 (TaqI), at a recombination fraction of θ = 0 (Nakamura et al. 1988).

Genotypic data with DSS346 for all of the 14 families requesting APC genotypic analysis are shown on pedigrees in figure 2. Primary data for 2 of the 14 pedigrees, APC-12 and APC-13, are shown in figure 3, with autoradiographs of the alleles and the scored genotypes. APC-12 (fig. 3A) is an APC kindred in which a deletion appears to segregate with the disease phenotype in the three known affected individuals (03, 04, and 08). This conclusion is based on the fact that, if affected individual 04 was a homozygote for the “1” allele, then his daughter, individual 06, should have inherited a “1” allele and could not have inherited the DSS346 allele(s) that is (are) shown. Individual 06 must have inherited the DSS346 “3” allele from her mother and the DSS346 deletion allele from her father (fig. 3A). Thus, the affected family members are likely hemizygous for DSS346. Parentage testing with markers at four VNTR loci showed with 99.91% confidence that both offspring (individuals 03 and 04) sampled were biologically related to the parent who carries the deletion, i.e., individual 08 (data not shown). Although it has not been determined whether the boundaries of this constitutional deletion exclude or include the APC gene, this deletion has been detected with other primer sets that were designed from sequences within 30–40 bp of the original primers, LNS-CAI and LNS-CAII; all combinations of primers (including an original and a new primer) detected the deletion (data not shown). None of the RFLP markers used in this study detected this particular deletion. With respect to presymptomatic diagnosis, gene carrier status was determined for the four at-risk individuals (01, 02, 06, and 07) in APC-12; only individual 06 had inherited the deletion.

APC-13 (fig. 3B) is an example of one of the clinical families in which the DSS346 marker was effective for the prediction of gene carrier status. The two individuals known to be affected (individuals 01 and 03) share a common DSS346 allele—allele 1—that segregates with the disease phenotype in this family. DSS346 was informative for the four at-risk members (individuals 05, 06, 07, and 08); again, only the female in generation III (individual 05) has inherited the APC-causing mutation.

Informativeness for seven polymorphic systems (five RFLP markers) in all 14 APC families is indicated in table 2. In 11 of the 14 APC families tested, the genotypic analysis was unambiguous; almost all of the persons at risk for developing the disorder could be determined as to whether they had inherited the mutant APC allele segregating in their families. In four of these families, however, the analysis was more complex. In APC-3, using a haplotype analysis of the informative two-allele systems, we initially determined that the affected individual (02) in generation II and/or the single individual (01) at risk for developing APC carry a chromosome 5 in which meiotic recombination has occurred (data not shown). Although we cannot be sure
Figure 2
whether the recombination event occurred proximal to or distal to the APC gene, we assume that no recombination took place between DSS346 and APC. Therefore, DSS346 allows the prediction of gene carrier status for the single at-risk individual (01), since the two known affected individuals (02 and 03) share a DSS346 allele. In APC-4, a prediction could not be made for the two presumptively at-risk individuals (03 and 04), because of nonpaternity; the unaffected parent (individual 01) shown is the father of only the second female in generation II (individual 04). The biological father of the known affected individual in the second generation (individual 05) and the biological father of the other at-risk female (individual 03) were not available for testing. Although the misinheritances due to nonpaternity were not detected with DSS346, they were detected with other markers, pL5.79-2 (MspI) and pL5.71-3 (MspI). Medical records and parentage testing with four VNTR markers not on chromosome 5q also confirmed the nonpaternity, to a confidence level of 95.5% (data not shown). In APC-8, the affected female in generation II (individual 04) requested preconceptual testing so that the disease allele she had inherited could be assessed for informativeness. Since her affected parent was deceased and since both she and her affected sibling (individuals 04 and 03, respectively) have inherited the same two DSS346 alleles present in the unaffected parent (individual 02), DSS346 was not informative in this family. Three polymorphic systems proximal to DSS346 (and to the APC gene) were informative for the prediction of gene carrier status for any potential offspring of individual 04. In APC-10, although affected individuals in this family have an APC phenotype, the two known affected individuals (01 and 07) do not share any DSS346 alleles. All of the other markers were uninformative in this family, except for pL5.71-3 (MspI), which predicted that three of the at-risk individuals in generation III (individuals 03, 09, and 11) would have inherited the APC allele, as the disease appears to segregate with the 4.5-kb, or second, allele in the affected brother and sister in generation II (data not shown). Since DSS346 is closer to the APC gene than is pL5.71-3, we assume that the genotypes at the DSS346 locus more accurately reflect the APC gene carrier status. Parentage testing with additional VNTR markers indicated that individuals 01 and 07 are both offspring
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of individuals 05 and 06, with a probability of greater than 99.99% (data not shown). Therefore, we were not able to make any predictions of APC gene carrier status in APC-10.

The most informative of the two-allele systems, pL5.71-3 (MspI), was informative for five at-risk individuals in the 14 small families tested. In contrast, APC gene carrier status was determined with D5S346 in 20 of 31 at-risk individuals. The overall informativeness for the determination of APC gene carrier status with all seven polymorphic systems was 21 of 31 at-risk individuals.

**Discussion**

DNA markers that were available previously for the presymptomatic diagnosis of APC are not highly informative; moreover, most of them lie at some distance from the APC gene. Therefore, until recently it has been difficult to accurately determine gene carrier status in APC families. We have shown here that a highly polymorphic marker, D5S346, which is very close to the APC gene, will enable the clinician to improve the accuracy of presymptomatic diagnosis. First, we demonstrated Mendelian inheritance in three CEPH reference families. Second, we used D5S346 to show an increase, of at least 9.0 LOD units, in likelihood of linkage to the APC locus, in a large APC kindred; the large increase in statistical significance makes this marker extremely useful for linkage studies for research purposes.

Predictions of carrier status were easily made with the new DNA marker in most of the clinical families tested; carrier status could be determined for 20 of 31 at-risk individuals in 14 APC families. Some of the complexities and/or uncertainties encountered in the genetic diagnosis of inherited disorders appeared in 4 of the 14 families we tested—e.g., recombination between DNA markers and the gene of interest, nonpaternity, preconceptual diagnosis in which couples request the information for reproductive decisions, and possible linkage heterogeneity. For APC-3, we were able to predict that the at-risk individual was an APC gene carrier, even though she was a recombinant with DNA markers farther from the APC gene than is D5S346. Nonpaternity, a common problem in clinical testing, prevented predictions of gene carrier status to be made in APC-4 because neither the biological father of the affected offspring nor the biological father of one of the at-risk individuals was available for testing. The affected individual in APC-8 requested preconceptual diagnosis in order to plan for possible pregnancy; although D5S346 was not informative, three of the other polymorphic systems tested were informative for the prediction of gene carrier status. If mutation at the D5S346 locus itself is excluded, then there are at least three possibilities that could explain the observation that the two affected individuals...
in APC-10 do not share a common DSS346 allele. First, a recombination event could have occurred between the marker locus and the APC gene in one of the affected siblings. We believe that this is highly unlikely, because the marker is at most 70 kb from the APC gene. Second, two different mutant APC alleles could be segregating in this family; this could have occurred if each parent of the two affected offspring carries a different constitutional APC mutation, or if one parent carries an APC mutation and if a different somatic mutation in APC has occurred in the germ line of the other parent, or if two different somatic mutations in APC have occurred in the germ line of one or both of the parents. Last, the APC phenotype in one or both siblings could reflect mutation at a locus other than APC; that is, it could reflect linkage heterogeneity. However, we are unaware of any families with an APC phenotype, in which the mutant allele does not map to the APC locus on 5q.

The ability to detect a mutant allele that predisposes individuals in APC families to colon cancer is very important. Using the DSS346 CA-repeat marker, we now have a greater degree of confidence in predicting APC gene carrier status. A heterozygosity of approximately 83% makes DSS346 likely to be informative in most families tested, and, because it is only 30–70 kb from the APC gene, recombination events between the marker and APC should be very rare. If the marker is informative in a family and carrier status can be ascertained, then individuals who inherit a mutant APC gene can be monitored regularly by colonoscopy. We propose that the DSS346 marker be used initially for clinical diagnostic purposes; if affected individuals are not informative at this locus, then other polymorphic markers can be tested, including those listed in table 1. There are at least three new multiallelic dinucleotide (CA)-repeat markers on 5q that are potentially useful for the diagnosis of APC gene carrier status: one is within the MCC gene, approximately 150–200 kb from the APC gene (van Leeuwen et al. 1991a). The other two CA repeats are farther from APC; the first was isolated and characterized from DNA marker YN5.64 (Breukel et al. 1991), and the second has not been precisely localized but is believed to be between the centromere and YN5.64 (van Leeuwen et al. 1991b; C. Breukel, personal communication).

Inherited forms of colorectal cancer that map to the APC locus on chromosome 5 include APC (Bodmer et al. 1987; Leppert et al. 1987; Nakamura et al. 1988) and a variant of APC with heterogeneity in numbers of polyps and later-age onset, called “attenuated APC” (Leppert et al. 1990; Spirio et al. 1992). Presently, the exact

### Table 2

<table>
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<tr>
<th>FAMILY</th>
<th>NO. OF INDIVIDUALS SAMPLED</th>
<th>NO. OF KNOWN AFFECTED SAMPLED</th>
<th>NO. AT RISK</th>
<th>pL5.79-2 MspI</th>
<th>pL5.79-2 PstI</th>
<th>pL5.71-3 MspI</th>
<th>DSS346 PCR</th>
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</tr>
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<td>4</td>
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<tr>
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<td>4</td>
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<tr>
<td>Total</td>
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<td>34</td>
<td>31</td>
<td>4/31</td>
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<td>5/30</td>
<td>20/31</td>
<td>1/26</td>
<td>3/30</td>
<td>1/30</td>
</tr>
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</table>

* RC = recombinant at this locus; NA = data not available.
proportion of colon cancers that are attributable to inherited lesions in the APC gene is unknown. A number of inherited colon-cancer predispositions have yet to be mapped to a specific genetic locus; among those are hereditary nonpolyposis colon cancer, which includes Lynch syndromes I and II (Lynch et al. 1985a, 1985b). Families also exist in which the appearance of one or a few adenomatous colonic polyps appears to show an autosomal dominant mode of inheritance (Burt et al. 1985; Cannon-Albright et al. 1988). This new marker will help in ascertaining which, and how many, different types of colon-cancer predispositions map to the APC locus on 5q.

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