Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 4166–4170, May 1993
Genetics

Identification of a colon mucosa gene that is down-regulated in colon adenomas and adenocarcinomas

(colorectal tumorigenesis/tumor-suppressor gene/cDNA subtraction)

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Communicated by Robert C. Gallo, January 1, 1993 (received for review May 1, 1992)

ABSTRACT **A cDNA, which we call DRA (for down-regulated in adenoma) has been isolated. Its mRNA is expressed exclusively in normal colon tissue, probably only in the mucosal epithelia. Expression of the DRA gene is significantly decreased in adenomas (polyps) and adenocarcinomas of the colon. The DRA gene appears to be a single-copy gene present on chromosome 7, a chromosome associated with colorectal tumorigenesis. The predicted DRA polypeptide is an 84,500-Da protein that contains charged clusters of amino acids, primarily at the NH_2 and COOH termini. Together with potential nuclear targeting motifs, an acidic transcriptional activation domain, and a homeobox domain, these elements suggest a transcription factor or a protein that may interact with transcription factors. Such a function may be consistent with a role in tissue-specific gene expression and/or as a candidate tumor-suppressor gene.**

Colorectal tumorigenesis is a multistep process involving the loss of function of so-called tumor-suppressor genes, as well as the activation of oncogenes (1, 2). It is also marked by several phenotypically distinct stages during progression; these include normal, hyperplastic, benign, carcinoma, and metastatic stages. As such, it is an exceptionally useful paradigm for studying the molecular genetic basis of cancer.

Ras and *myc* have been found to be activated and/or show elevated expression in colorectal tumors. About half of large adenomas and at least half of carcinomas contain activated K-*ras* genes (3–5). c-*myc* overexpression (6, 7) and occasional gene amplification (8) have also been demonstrated in colorectal tumors. Furthermore, deregulated c-*myc* expression can be suppressed by microcell-mediated transfer of chromosome 5, the locus for the putative tumor-suppressor genes *APC* (for adenomatous polyposis coli) and *MCC* (for mutated in colorectal carcinoma) (9). In addition, several tumor-suppressor genes have been implicated in colorectal tumor progression. An allele of the p53 tumor-suppressor gene (chromosome band 17p13) is lost in many colon carcinomas (although not as much in adenomas) coupled with a point mutation in the remaining allele (10, 11). Importantly, transfection of a wild-type p53 gene into colon cancer cell lines *in vitro* has been shown to suppress cell growth, thereby demonstrating that the p53 gene product directly affects one major cancer characteristic (12).

The *DCC* (for deleted in colorectal carcinoma) gene (chromosome band 18q21) is also lost in most colon carcinomas and ≈50% of late adenomas. A portion of the *DCC* gene bears a homology to the neural cell adhesion molecule (N-CAM) (13). This result suggests that the *DCC* gene product may play a role in cell–cell contacts. Two genes, *APC* (14, 15) and *MCC* (16), have been mapped to a locus linked to the inherited disorder, adenomatous polyposis coli (APC; chromosome band 5q21). Both genes contain mutations/deletions in colon carcinoma; however, *MCC* mutations are not common among tumors, whereas the *APC* lesions are more common and found in the germ-line genomic DNA of APC patients (16, 17). It is notable that transfer of chromosome 5 to colon cancer cells lacking a normal *APC* gene suppresses tumorigenicity (18). This result reinforces the concept that *APC* and/or *MCC* are tumor-suppressor genes.

We describe here an additional candidate tumor-suppressor gene called DRA‖ (for down-regulated in adenoma), which maps to chromosome 7. Although chromosome 7 abnormalities associated with colorectal carcinoma have been seen (2, 19), chromosome 7 has not previously been described as a locus for a colon cancer tumor-suppressor gene. This cDNA clone was isolated by subtractive hybridization, a technique that does not require extensive information, such as loss of heterozygosity to identify candidate tumor-suppressor genes. The DRA gene product encodes a highly hydrophobic protein with charged clusters located particularly in the COOH terminus. Its expression appears strictly limited to the mucosa of normal colon, and it is down-regulated early in tumorigenesis.

MATERIALS AND METHODS

DRA cDNA Isolation. Construction of the cDNA libraries in λZAP II from normal colon and adenocarcinoma tissues and cDNA subtraction have been described (20). Subtracted single-stranded cDNA inserts were amplified by PCR using the KS and SK sequencing primers supplied by the manufacturer (Stratagene). Amplified cDNA was labeled with ^{32}P by the method of Feinberg and Vogelstein (21), except that the KS primer was used as a specific primer, rather than using random primers. Differential plaque hybridization was done with the enriched normal and adenocarcinoma probes on duplicate lifts from a total of 5×10^5 plaques of normal colon λZAP II library in the presence of unlabeled pBluescript DNA at 10 μg/ml. Plaques that hybridized differentially with the two probes were further purified and rescued as pBluescript phagemid clones, according to the manufacturer's protocol (Stratagene). One such clone, 611, was used to reprobe the normal library to find full-length cDNAs. The full-length sequence is hereafter referred to as DRA. Sequence analyses (nucleotide and protein) were done on the

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Abbreviations: APC, adenomatous polyposis coli; DCC, deleted in colorectal carcinoma; DRA, down-regulated in adenoma; MCC, mutated in colorectal carcinoma.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. L02785).

Genetics Computer Group package at the Advanced Scientific Computer Laboratory, Frederick, MD (22).

Cell Lines and Tissues. The VACO235 and VACO330 colon adenoma cell lines were from J. K. V. Willson (Case Western Reserve University). All other cell lines were purchased from the American Type Culture Collection. Human tissues were procured from several sources: The Cooperative Human Tissue Network, Mt. Sinai Medical Center (S. Suster), and from A. Weston (National Cancer Institute, Laboratory of Human Carcinogenesis).

RNA Isolation and Analysis. RNA from cultured cells was purified by centrifugation through a CsCl cushion, according to the method of Chirgwin *et al.* (23). Tissue samples were ground to a powder under liquid nitrogen, then lysed, and centrifuged as above (23). Total RNA was fractionated on 1.2% agarose gels containing 0.66 M formaldehyde (2.2 M in the sample) by the method of Lehrach *et al.* (24). Gels were transferred either to nitrocellulose [in 20× SSPE (1× SSPE is 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)] or to GeneScreen (in 0.1 M sodium phosphate, pH 6.5).

Chromosome Assignment. A central *Eco*RI fragment of the DRA cDNA was hybridized to two panels of somatic cell hybrid genomic DNAs purchased from Bios (New Haven, CT). The hybridization results to each panel were scored blindly and separately from one another.

RESULTS

Isolation of DRA cDNA. Primary cDNA libraries and subsequent subtractions were made from matched normal colon and colon carcinoma, as described (20). Inserts from the two subtraction libraries were subsequently amplified by PCR using the KS and SK sequencing primers designed for the pBluescript vector. These amplified inserts, in turn, were labeled with ^{32}P and used as enriched cDNA probes to screen the primary cDNA libraries by differential hybridization. Of 500,000 plaques screened, four showed a strong differential hybridization in favor of the normal-enriched probe. All were confirmed as differential by using them as probes to RNA blots of normal and tumor tissue. With clone 611, additional clones containing the full coding sequence were isolated by conventional plaque hybridization. One of these clones, now called DRA, was further analyzed and is described herein.

Expression of DRA in Adenocarcinoma. The DRA clone was originally isolated from a normal colon tissue cDNA library using the subtractive hybridization technique (20). It is possible that loss of expression of this sequence was particular to the tumor sample used to construct the cDNA libraries. To assess whether loss of expression was a general phenomenon of colon adenocarcinomas, a battery of matched (i.e., from the same patient) tumor and normal tissues were analyzed by RNA blot hybridization for DRA mRNA expression. Fig. 1*A* shows pairs of matched adenocarcinoma and normal tissues. In eight of nine such matched samples, the tumor tissues are completely lacking in expression of the single 3.2-kb DRA mRNA. Although all normal colon samples tested express DRA mRNA, it is also noted that the absolute level of DRA expression can vary widely from sample to sample. By hybridizing the DRA cDNA back to the normal colon cDNA library, the amount of DRA mRNA was estimated to range from 0.01% to 0.1% of the mRNA population. With one exception, all tumor samples show a marked reduction in the amount of DRA mRNA expressed; sample 9 still retains a high level of expression in the tumor piece. This tumor is not remarkable for any differences from the other adenocarcinoma samples, which include both right and left colon and well-differentiated to poorly differentiated specimens. However, the tumor specimens from sample 9 could also include a significant portion of normal tissue, which would account for the continued presence of DRA mRNA in the total RNA isolated from that tumor sample.

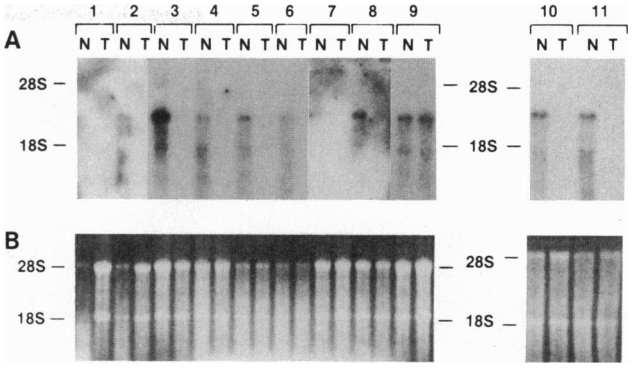

FIG. 1. RNA hybridization to matched normal and carcinoma tissues. Five micrograms of total RNA was fractionated on a 1.2% agarose-formaldehyde gel and then transferred to a nitrocellulose filter. Matched samples (lanes 1–9) are bracketed. All tumor (T) samples are adenocarcinoma. The normal (N) tissue in lanes 5, 6, 10, and 11 is from the mucosal epithelia dissected from its underlying tissues. (*A*) Hybridization to the RNAs with a 480-bp probe derived from the 5′ end of the DRA cDNA (nt 11–481 from Fig. 6). Autoradiography for lanes 1, 2, 7, 8, 10, and 11 was done overnight; lanes 3, 4, 5, 6, 7, and 9 were exposed for 3 days. (*B*) The corresponding EtBr-stained gel.

It is also noteworthy that DRA expression occurs in the mucosal layer of normal colon; the mucosal layer typically is the origin of colonic neoplasms. In matched samples 5 and 6, the normal tissue consists of only mucosal layer that had been dissected away from the underlying tissues. Similarly, lanes 10 and 11 show additional normal mucosa along with tumor specimens; however, these are not matched samples. Nonetheless, normal mucosa is shown to express DRA mRNA, whereas tumor tissue does not express DRA mRNA. At this point, we could not rule out the possibility that layers under the mucosa may also express DRA (however, see below). Fig. 1*B* shows that the amount of RNA loaded in each lane is approximately the same. The normal RNA for lanes 1 and 2 is somewhat degraded, and this may explain the relatively low expression of DRA seen in the corresponding lane of Fig. 1*A*.

Expression of DRA in Adenomas. Benign adenomas, usually polyps, were also analyzed for DRA mRNA expression by RNA blot analysis. Fig. 2*A* shows that adenomas also show a significant decline or absence of the 3.2-kb DRA mRNA relative to normal tissue. Again, whether the small amounts of expression seen in some adenoma samples (lanes 2 and 4) derive from adenoma cells or residual normal mucosa is unclear. Interestingly, the villous adenoma-derived cell line, VACO235 (lane 7), still expresses low, but detectable, amounts of DRA mRNA. Because it is a cell line, the expression seen here cannot be from contaminating normal mucosa. Another adenoma-derived cell line, VACO330, does not express detectable DRA mRNA (Fig. 3*A*) compared with VACO235 or normal tissue. However, doubling the RNA amount in lane 2 (Fig. 3) allowed us to see some trace amount of DRA expression in VACO330 cells (data not shown). Figs. 2*B* and 3*B* indicate that the amount and quality of RNAs loaded in each lane are generally similar, except for Fig. 3*B*, lane 2, where there is evidence of some degradation.

Expression of DRA in Other Tissues. We examined DRA expression in a number of human tissues. Fig. 4 shows that only normal colon expresses significant quantities of DRA mRNA. In addition to primary tissues, a number of cell lines from normal tissues were also tested (Fig. 5). Similarly, none of these cells expressed significant quantities of DRA mRNA. Of particular note is cell line HISM (lane 7), which is derived

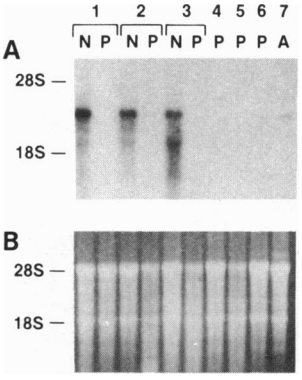

FIG. 2. RNA hybridization to adenoma cells. Five micrograms of total RNA was fractionated on a 1.2% agarose-formaldehyde gel and then transferred to a nitrocellulose filter. Matched samples of normal (N) tissue and benign polyp (P) are bracketed (lanes 1–3). Lanes 4–6 are from unmatched polyp (P) tissue. Lane 7 is from the benign adenoma (A) cell line VACO235. (*A*) Hybridization to the RNAs with a 480-bp probe derived from the 5′ end of DRA cDNA. Autoradiography was done overnight. (*B*) The corresponding EtBr-stained gel.

from intestinal smooth muscle and CCD18Co (lane 3), CCD33Co (lane 4), and CCD112CoN (lane 5), which are fibroblast cells derived from normal colon. These cells are all derived from regions other than the mucosal epithelia of normal colon, and they do not express DRA mRNA. CCD841CoN (lane 2), while epithelial-like in morphology, lacks any definitive epithelial characteristics (e.g., it does *not* stain for keratin), so it cannot be concluded to have derived from mucosal epithelia. Therefore, expression of DRA mRNA is apparently restricted to normal colon and probably to the mucosal layer, in particular.

The DRA Sequence. The significant features of the DRA gene product, as deduced from its nucleotide sequence, are presented in Fig. 6. The cDNA (2882 nt) contains an open reading frame of 764 amino acids, including the initiation methionine. There are two in-frame stops before the initiation methionine and nine stops after the TAA codon. A polyadenylylation signal, ATTAAA, is found 24 nt upstream from the poly(A) tail. The sequence around the initiation methionine is TCAAAATGA, which does not conform to the Kozak consensus sequence (25) of CCRCCATGG for initiation. However, because two in-frame stops precede this methionine and because the next methionine is encoded within the sequence CTGAGTATGA, there is no more likely candidate for initiation. The sequence does contain the crucial adenine residue at position −3. However, the guanine at position +4 of the consensus, which is also an important residue for translation initiation, does not exist in the DRA sequence; rather, it is replaced by adenine. Nonetheless, of 699 sequences com-

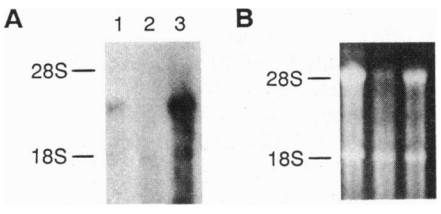

FIG. 3. RNA hybridization to adenoma cell lines. Five micrograms of total RNA was fractionated on a 1.2% agarose-formaldehyde gel and then transferred to a nitrocellulose filter. Lanes: 1, villous adenoma cell line VACO235; 2, tubular adenoma cell line VACO330; 3, normal colon tissue. (*A*) Hybridization to the RNAs with a 480-bp probe derived from the 5′ end of the DRA cDNA. Autoradiography was done overnight. (*B*) The corresponding EtBr-stained gel.

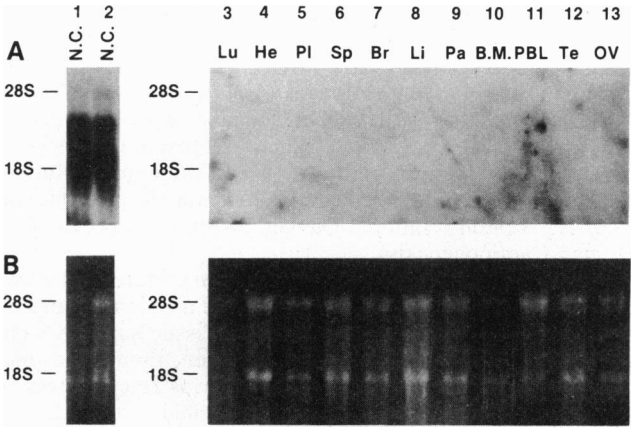

FIG. 4. RNA hybridization to normal tissues. Five micrograms of total RNA was fractionated on a 1.2% agarose-formaldehyde gel and then transferred to a GeneScreen filter. Lanes: 1 and 2, normal colon (N.C.) tissue; 3, lung (Lu); 4, heart (He); 5, placenta (Pl); 6, spleen (Sp); 7, brain (Br); 8, liver (Li); 9, pancreas (Pa); 10, bone marrow (B.M.); 11, peripheral blood leukocytes (PBL); 12, testis (Te); 13, ovary (OV). (*A*) Hybridization to the RNAs with a central fragment of DRA cDNA. Autoradiography was done for 4 days. (*B*) The corresponding EtBr-stained gel.

piled by Kozak, 114 functional initiator codons contain adenine residues at positions −3 and +4. In fact, the human α-amylase and α-lactalbumin mRNAs exactly match the sequence of the DRA mRNA at positions −4 to +4 (26). Finally, it cannot be ruled out that a GTG codon at amino acid Val-11 could be used as a non-ATG initiation site (27).

A search of the GenBank and European Molecular Biology Laboratory nucleotide data bases failed to reveal any sequences to which DRA has any significant homology. Nor were any significant homologies found when the open reading frame was used to search the protein data bases. The predicted 84,504-Da DRA protein contains clusters of charged amino acid residues at its NH_2- and COOH-terminal regions, particularly at the COOH terminus, starting from amino acid Arg-460. The central region (amino acids Val-176–Gly-459) is largely hydrophobic, although it is occasionally interrupted by islands of charged clusters. Casein kinase II and phos-

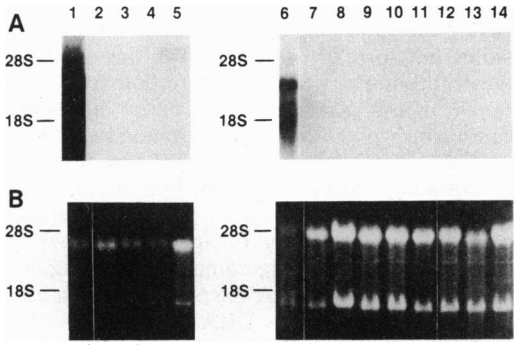

FIG. 5. RNA hybridization to normal cell RNAs. Five micrograms of total RNA was fractionated on a 1% agarose-formaldehyde gel and then transferred to a GeneScreen filter. Lanes: 1, normal colon tissue; 2, CCD841CoN (normal colon cells, epithelial-like); 3, CCD18Co (normal colon fibroblasts); 4, CCD33 (normal colon); 5, CCD112CoN (normal colon fibroblasts); 6, normal colon tissue; 7, HISM (human intestinal smooth muscle); 8, RPMI 7666 (lymphoblasts); 9, HS67 (thymus); 10, FHS738.Bl (bladder); 11, WI-38 (lung); 12, Detroit 55 (skin); 13, HBL-100 (breast epithelia); 14, Hs1.Tes (testis). (*A*) Hybridization to RNAs with a 5′ fragment derived from the DRA cDNA. Autoradiography was done overnight for lanes 1–5 and for 4 days for lanes 6–14. (*B*) The corresponding EtBr-stained gel.

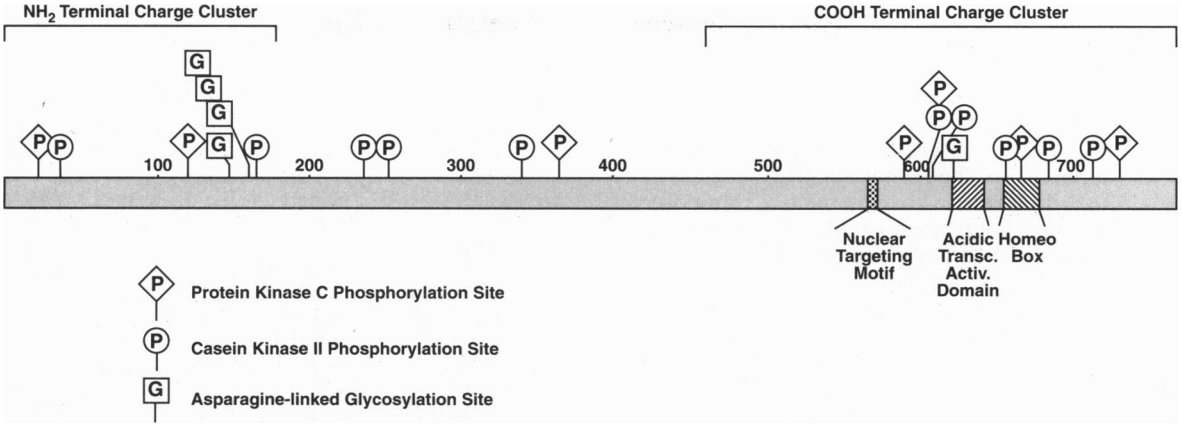

FIG. 6. DRA protein as deduced from its nucleotide sequence. The numbers indicate amino acid position. The complete sequence is available in GenBank. Trans. Activ., transcriptional activation.

phokinase C phosphorylation sites are also clustered predominantly within the COOH-terminal region (Arg-460–Phe-764), whereas asparagine-linked glycosylation sites are almost all (four of five) with the NH_2-terminal region (Met-1–Arg-175).

Three potential nuclear-targeting sites are indicated in Fig. 6, with one, in particular (Arg-569–Lys-573), very closely conforming to the consensus (R/K/T/A)KK(R/Q/N/T/S/G)K (28). A conservative Arg → Lys substitution is the only change found. The other two potential nuclear-targeting sites have a less conservative single-amino acid substitution (see Fig. 7*A*). A homeobox motif at amino acids Phe-653–Arg-676 mismatches the consensus by only a single conservative substitution (Trp-667 → Leu). Finally, a possible acidic transcriptional activation domain exists at amino acids Ile-620–Glu-640. The significance of these motifs will be discussed below.

Chromosomal Localization. A central *Eco*RI fragment of the DRA cDNA was used to hybridize to a genomic blot of two panels of somatic cell hybrids. Both panels indicated that the DRA gene is located on chromosome 7. There was 100% concordance for chromosome 7 and 100% discordance for all other chromosomes.

DISCUSSION

We describe the isolation of a cDNA clone for another tumor-suppressor candidate gene. Because expression from this gene is lost early in tumorigenesis, we call the gene DRA. Among the candidate suppressor genes involved in colorectal cancer, p53 is widely expressed in other tissues (11). The *DCC* gene is expressed at higher levels in the brain than in normal colon (13), and the *MCC* and *APC* gene products are expressed in many tissue types (15, 16). In contrast, our analysis with DRA indicates a very restrictive pattern of expression. In fact, only tissue derived from the mucosa of the colon appears to express significant levels of the DRA mRNA. Although the DRA mRNA was readily detected in most normal samples, some normal tissues had much lower levels (although still higher than in tumor). This observation may conform to that of Augenlicht *et al.* (29), which showed that the flat "normal" mucosa of patients at risk for APC or hereditary nonpolyposis colorectal cancer shows molecular expression changes similar to tumor tissue.

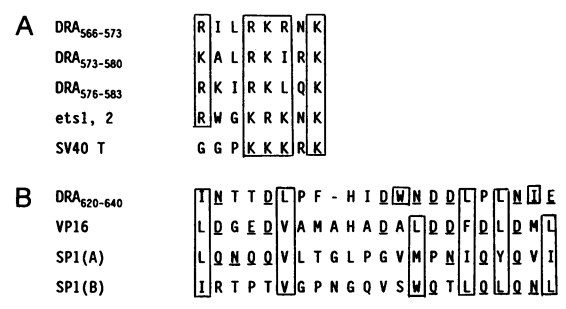

FIG. 7. (*A*) Alignment of nuclear targeting motifs. SV40 T, simian virus 40 large T antigen. (*B*) Alignment of acidic transcriptional activation domains. Hydrophobic amino acids are boxed, and amino acids with carbonyl-containing side groups are underlined.

The DRA gene was mapped to chromosome 7. This chromosome has not been previously associated with tumor progression in colorectal carcinoma through gene loss. However, Paraskeva *et al.* (2) have reported both polysomies and monosomies of chromosome 7 in various cultured colorectal cell lines. Polysomies of chromosome 7, as well as breakpoints at chromosome 7p in colon carcinoma cells, have been reported by other investigators as well (19). Interestingly, the only adenoma sample in which we observed DRA expression, VACO235, contains a translocation of "extra material" to chromosome 7q. In contrast, the adenoma cell line VACO330, which does not express DRA, has a normal diploid karyotype (30). We did not detect any gross rearrangements of DRA genomic DNA in VACO235 or in any of several colon carcinoma cell lines tested (data not shown). Therefore, the mechanism for the loss of DRA expression is more subtle.

The fact that DRA expression is down-regulated in adenomas and adenocarcinomas may not be due to mutational inactivation at all. Rather, an epigenetic mechanism may apply. Although general hypomethylation of the genomic DNA is observed early in colorectal tumorigenesis (31, 32), DNA methyltransferase transcription has been shown to be increased 15 times in normal-appearing mucosa around benign tumors (33). Much higher levels of expression are seen in premalignant polyps (60-fold increase), and even higher levels (200-fold) are reported in adenocarcinomas. This result suggests a mechanism whereby down-regulation of the DRA gene may be achieved through specific methylation of CpG sites, presumably in the 5′-regulatory regions of the gene.

The predicted protein product of the DRA gene is an 84,500-Da polypeptide. The open reading frame has been verified by *in vitro* translation and SDS/PAGE of *in vitro*-transcribed mRNA (data not shown). Furthermore, polyclonal antibodies directed against several oligopeptides from the COOH-terminal one-third of the protein react with a truncated version of DRA expressed in *Escherichia coli* (data not shown). The deduced DRA polypeptide contains several noteworthy motifs suggestive of transcription factors or of proteins that interact with transcription factors. The COOH

and NH_2 ends of the protein contain numerous charged amino acid residues. Such charge clusters have been noted in functional domains of transcription factors (34). Within the COOH-terminal half are three potential nuclear localization signals (Fig. 7*A*). There is also an acidic region (Fig. 7*B*), which may serve as a transcriptional activation domain similar to that reported for the herpes simplex virus 1 VP16 protein (35). The distinguishing characteristic of this motif is that of "bulky" hydrophobic amino acids (shown in boxes) flanked by amino acids with carbonyl-containing side groups (shown underlined). In contrast, however, no leucine zipper or zinc finger motifs have been observed. Furthermore, we estimated the DRA mRNA to be ≈0.01–0.1% of the mRNA population. This percentage may be rather high for a transcription factor. By comparison, Sp1 composes ≈0.003% of HeLa cell protein (36), and Ap1 composes up to 0.005% of HeLa cell protein (37). Therefore, the suggestion that the DRA protein is a transcription factor or a protein that interacts with transcription factors remains tentative until empirical experimentation provides direct evidence.

The authors thank Laura Burdett, Robert J. Fisher, and Arun Seth for helpful discussions; Dennis Watson for critical review of the manuscript; Mark Gunnell and Gary Smythers at the Advanced Scientific Computing Laboratory for help with some of the Genetics Computer Group programs; and Karen Cannon for expert typing.

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