When *ARS307* is deleted from the chromosome, *ARS308* continues to be an inefficient origin; it does not take over the replication initiation function for this region of the chromosome (*12*). In this case, close spacing is clearly not the explanation for inactivation of *ARS308*. It is more likely that *ARS308* lacks sequence elements that are required for efficient initiation. It is not known over what distance efficient origins exert their interference. Nor is it known whether there might be a threshold distance for origin interference or a gradual increase in interference as origins are brought into closer proximity.

We speculate that if we were to add a third or a fourth ARS in close proximity to *ARS1* and *ARS501*, we would see further diminution of initiation at each of the ARSs. Consequently, there would be an increase in the number of replication forks passing through each ARS because of initiation at one of the flanking origins. In this region each restriction fragment that contains an ARS near its center would give the same 2D gel pattern—that is, a faint bubble arc with a very prominent complete simple Y arc. Two additional copies of *ARS1* have been integrated 3.5 kb apart at the normal locus of *ARS1* on chromosome IV (Fig. 5). It appears that the three copies of *ARS1* share the responsibility of initiating replication in this chromosomal region: simultaneous activations of adjacent origins are rare, and each copy of *ARS1* displays a faint bubble arc and a more prominent arc of simple Y's on 2D gels (Fig. 5). When overlapping fragments from the 55-kb initiation zone of the amplified DHFR locus of Chinese hamster ovary cells are examined on 2D gels (*14*), each fragment gives this composite pattern. It has been suggested that a broad initiation zone reflects the lack of specific origin sequences. However, we conclude that the data from the DHFR locus are also consistent with the close spacing of multiple, specific initiation sites that experience origin interference.

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Association of the *APC* Gene Product with β-Catenin

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Mutations in the human *APC* gene are linked to familial adenomatous polyposis and to the progression of sporadic colorectal and gastric tumors. To gain insight into APC function, APC-associated proteins were identified by immunoprecipitation experiments. Antibodies to APC precipitated a 95-kilodalton protein that was purified and identified by sequencing as β-catenin, a protein that binds to the cell adhesion molecule E-cadherin. An antibody specific to β-catenin also recognized the 95-kilodalton protein in the immunoprecipitates. These results suggest that APC is involved in cell adhesion.

Multiple genetic alterations, including mutations in *RAS* and in the tumor suppressor genes *APC*, *P53*, and *DCC*, contribute to the progression of colorectal tumorigenesis [reviewed in (*1*)]. Among these, *APC* mutations appear earliest in the pathway and are observed in small benign adenomas of the colon (*2*). Mutations in *APC* are also linked to an inherited form of colon cancer, familial adenomatous polyposis (*3*, *4*). The protein product of *APC* is a 2844–amino acid polypeptide containing a potential coiled-coil structure in the NH_2-terminal region, a repeated 20–amino acid sequence in the central region, and a stretch of basic amino acids in the COOH-terminal region (*3*). The *APC* mutations associated with cancer result in the production of APC proteins that are truncated at the COOH-terminus (*2*, *5*, *6*). How these deletions compromise APC function is unknown, as no functions have yet been ascribed to the protein, except for the homo-oligomerization domain localized to the extreme NH_2-terminus (*7*).

To characterize the APC protein, we generated polyclonal antisera against two separate regions of the polypeptide. One antiserum, anti-APC2, recognized epitopes in the central region of the protein, and the other, anti-APC3, was specific to the COOH-terminal region (Fig. 1A). The antibodies were tested on the colorectal cancer cell lines SW480, HCT116, and DLD-1, and the kidney cell line 293 (*8*). Anti-APC2 reacted with the full-length, wild-type APC from 293 cells and with mutant APC from SW480 cells (Fig. 1B). Full-length APC was immunoprecipitated from the 293 and the HCT116 cells by both anti-APC2 and anti-APC3. By contrast, only anti-APC2 recognized the mutant APC in the SW480 and DLD-1 cells. The single APC allele in the SW480 cells contains a nonsense mutation at codon 1338 (*9*) and produces a stable truncated form of APC (*6*). The DLD-1 cells also express a mutant APC similar in size to that detected in SW480 cells (Fig. 1B).

To identify proteins potentially associated with APC, we performed immunoprecipitations on lysates from cells metabolically labeled with [^{35}S]methionine. The antibodies to APC immunoprecipitated wild-type APC from the 293 and HCT116 cell lysates, but only the truncated APC from the SW480 cells (Fig. 2A). In addition to APC, a prominent radiolabeled protein of ~95 kD was present in anti-APC2 immunocomplexes from HCT116, 293, and SW480 cells. The 95-kD protein was also detected in anti-APC3 immunoprecipitates from HCT116 and 293 cells, but not from SW480 cells. This result demonstrated that the 95-kD protein is associated with APC and does not react directly with the antibodies to APC. Other polypeptides were also immunoprecipitated by antibodies to APC; however, most of these proteins appeared to bind nonspecif-

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ically, as they were also present in control immunoprecipitates.

The 95-kD protein associated with both wild-type APC and the truncated mutant, although the results of stringent washes indicated that it bound with less avidity to the mutant (Fig. 2B). To determine which region of APC bound the 95-kD protein, we added purified APC fragments to cell lysates and then retrieved them with an antibody specific to an epitope tag engineered onto each fragment. Interestingly, this "fishing" technique was only successful when lysates were prepared from cells that contained mutant APC protein, such as SW480 cells (Fig. 2B). The central region of APC, represented by the APC2 fragment (Fig. 1A), stably associated with the 95-kD protein in the SW480 cell lysate, whereas the COOH-terminal fragment, represented by APC3, did not form a complex. APC4, which contains both the APC2 and APC3 regions, was equivalent to APC2 in binding the 95-kD protein. The 95-kD protein could not be recovered from 293 cell lysates through its association with exogenously added APC.

We purified the 95-kD protein from SW480 cell lysates by using immobilized recombinant APC2 as an affinity matrix (Fig. 2C). After SDS–polyacrylamide gel electrophoresis (PAGE), the affinity-purified 95-kD protein was excised and then digested in situ with Glu-C protease during electrophoresis in a second gel. Proteolytic fragments, blotted to a polyvinylidene difluoride (PVDF) filter, were then directly subjected to amino acid sequencing. One fragment yielded the sequence Gln-Asn-Ala-Val-Arg-Leu-His-Tyr-X-Leu-Pro-Val-X-Val-Lys (where X denotes undefined residues), which is also found at positions 482 to 496 of human β-catenin (*10*). A second fragment yielded another β-catenin sequence, Asn-Asp-Glu-Asp-Gln, found at position 161 to 165. These partial sequences distinguish the 95-kD protein from plakoglobin, the closest known vertebrate relative of β-catenin (*11*).

To confirm the identity of the 95-kD protein as β-catenin, we analyzed the anti-APC immunoprecipitates on protein blots developed with a rabbit polyclonal antibody to β-catenin (Fig. 3A). This antibody recognized a 95-kD polypeptide immunoprecipitated by anti-APC2 from the 293, SW480, and HCT116 cell lysates. Furthermore, anti-APC3 immunoprecipitated β-catenin from 293 and HCT116 cell lysates, but not from SW480 cell lysates. Trace amounts of protein reactive with the antibody to β-catenin were detected in the anti-APC3 immunocomplex from SW480 cells, but these proteins were also present in control immunoprecipitates and therefore are likely due to nonspecific binding.

We next used anti-APC2 to probe blots of proteins immunoprecipitated by the antibody to β-catenin. Mutant APC was clearly detected in the immunoprecipitate from the SW480 cell lysate, and wild-type APC was detected in the immunoprecipitate from the 293 cells (Fig. 3B). Because crude rabbit serum was used for immunoprecipitation, detection of wild-type APC was partially obscured by the high background in that region of the blot.

β-catenin associates with the cytoplasmic tail of the cell adhesion molecule E-cadherin, also known as uvomorulin, and is thought to be essential for the normal function of E-cadherin in maintaining the adherens junction of epithelial cells (*12, 13*). Because β-catenin associates with E-cadherin and with APC, it is possible that APC is also a component of a multimeric E-cadherin complex. To investigate this possibility, we analyzed E-cadherin immunoprecipitates for the presence of APC, and, conversely, APC immunoprecipitates for the presence of E-cadherin. In both cases, the results were negative (Fig. 4). A cadherin-APC complex could, nevertheless, exist but go undetected because of the relative sensitivity of the antibodies to E-cadherin and APC. However, as these two antibodies precipitated comparable amounts of β-catenin (Fig. 4B), it seems more likely that

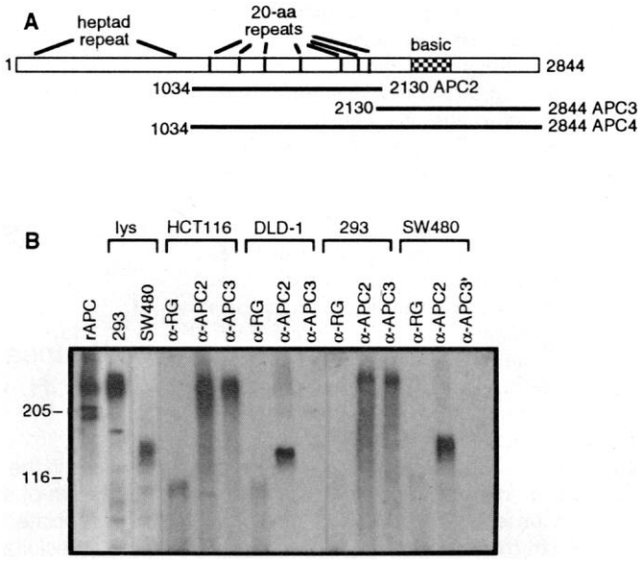

Fig. 1. Characterization of antibodies to APC. (**A**) Linear representation of wild-type APC. The general structural features of APC (*3*) are indicated at the top and the APC protein fragments used in the present study (*16*) are aligned below. (**B**) A protein blot developed with anti-APC2. The lane labeled "rAPC" contains 5 ng of full-length recombinant APC and that labeled "lys" contains 50 μg of total protein from 293 or SW480 cell lysates. The remaining lanes contain proteins immunoprecipitated (*17*) with a control antibody to the rap1 guanosine triphosphatase activating protein (GAP) (α-RG) or with the indicated affinity-purified antibody to APC. The positions and molecular sizes (in kilodaltons) of prestained protein standards are shown to the left: myosin, 205; β-galactosidase, 116.

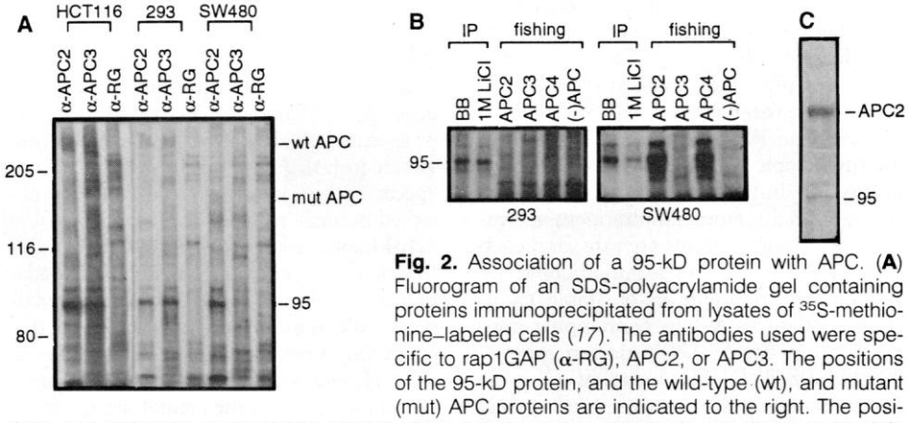

Fig. 2. Association of a 95-kD protein with APC. (**A**) Fluorogram of an SDS-polyacrylamide gel containing proteins immunoprecipitated from lysates of ^{35}S-methionine–labeled cells (*17*). The antibodies used were specific to rap1GAP (α-RG), APC2, or APC3. The positions of the 95-kD protein, and the wild-type (wt), and mutant (mut) APC proteins are indicated to the right. The positions and molecular sizes (in kilodaltons) of prestained protein standards are shown to the left: myosin, 205; β-galactosidase, 116; bovine serum albumin, 80. (**B**) Fluorogram of polypeptides associated with recombinant APC added to ^{35}S-methionine–labeled cell lysates. "Fishing" denotes the addition of purified epitope-tagged APC fragments to cell lysates with subsequent immunoprecipitation with a monoclonal antibody specific to the epitope tag (*18*). For comparison, endogenous APC was directly immunoprecipitated (IP) from the same lysates with anti-APC2. These immunoprecipitates were washed with buffer B alone (BB) or with buffer B containing 1 M LiCl. (**C**) A Coomassie blue–stained SDS-polyacrylamide gel showing proteins obtained from the lysate of one T-150 flask of confluent SW480 cells by "fishing" with 20 μg of APC2 protein.

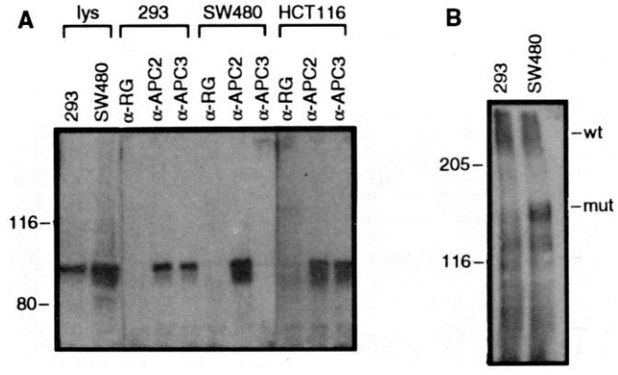

Fig. 3. Analysis of the 95-kD protein with an antibody to β-catenin. (**A**) A protein blot developed with an antibody to β-catenin. The lanes labeled "lys" contain 50 μg total protein from 293 or SW480 cell lysates. The remaining lanes contain proteins immunoprecipitated with a control antibody to rap1GAP (α-RG) or with the indicated antibody to APC. Immunoprecipitations were performed as in Fig. 1B (*17*). (**B**) A protein blot developed with anti-APC2. Lanes contain immunoprecipitates obtained with the antibody to β-catenin. The positions of mutant (mut) and wild-type (wt) APC proteins are indicated. Molecular size standards are as in Fig. 2.

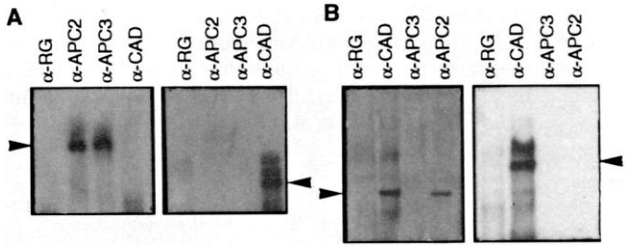

Fig. 4. Analysis of immunoprecipitates for the association of E-cadherin with APC. Lanes contain immunoprecipitates obtained with antibodies to rap1GAP (α-RG), APC2, APC3, or E-cadherin (α-CAD). (**A**) A protein blot of immunoprecipitates from 293 cell lysates developed with an antibody to APC2 (left) or to E-cadherin (right). Arrowheads indicate positions of APC and cadherin. (**B**) A protein blot of immunoprecipitates from SW480 cell lysates developed with an antibody to β-catenin (left) or to E-cadherin (right). Arrowheads indicate positions of β-catenin and cadherin.

β-catenin forms independent complexes with either APC or E-cadherin.

To investigate whether APC and E-cadherin share a β-catenin-binding motif, we compared their amino acid sequences. Although no significant identities were found, the sequence Ser-Leu-Ser-Ser-Leu is contained in the catenin-binding domain of E-cadherin (*13*) and is also part of the consensus sequence derived from the 20–amino acid repeat contained in APC (*3*). All seven copies of this imperfect repeat are contained within the APC2 fragment, which binds β-catenin. Most of the truncated proteins predicted from APC mutations are devoid of at least five of these repeats and, in some cases, all seven.

We postulate that mutant APC proteins have a reduced affinity or no affinity for β-catenin and that this deficiency may contribute to the mutant APC phenotype. The formation of colorectal polyps associated with APC mutations may result from a loss of intercellular contact or communication that is dependent upon an interaction between APC and β-catenin. A possible role for β-catenin in cell signaling can be inferred from its homology to the product of the *Drosophila* segment polarity gene, *armadillo* (*14*). Armadillo is a downstream target of Wingless, a secreted protein factor that is thought to be a signaling molecule in pattern development (*15*).

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16. We constructed a composite full-length *APC* cDNA from cDNA libraries of human fetal brain, placenta, and heart (Stratagene). Four ^{32}P-labeled oligonucleotide probes, ranging in size from 245 to 287 base pairs (bp) were generated with oligonucleotide primers that were based on the *APC* nucleotide sequence (*4*). Screening with these probes resulted in the isolation of four overlapping cDNA clones encoding the entire *APC* gene product, including 357 bp of previously unreported 5′-untranslated sequence. Three of these clones were identical to the published *APC* sequence; the fetal brain clone contained silent mutations at nucleotides 4416 (A to G), 5034 (A to G), 5265 (G to A), 5268 (G to T), and 5880 (A to G). *APC* constructs 2, 3, and 4 were generated by subcloning convenient restriction fragments into pGEM (Promega) or pBlueScript (Stratagene), in which the polylinker was replaced with a synthetic linker engineered to encode an initiating methionine, the Glu-Glu epitope tag (*19*) and, when necessary, additional nucleotides to complete the construct. The epitope-tagged gene fragments were subcloned into the baculovirus transfer vector pAcC13 for expression in sf9 insect cells (*19*). Recombinant baculovirus containing the desired cDNA inserts were used to infect sf9 cells and the APC proteins purified by anti-Glu-Glu affinity chromatography (*20*).
17. Polyclonal antisera to APC were raised by an initial subdermal injection of 0.5 mg of each APC fragment per rabbit, followed by three boosts of 0.25 mg each at 2-week intervals. Antibodies were purified by affinity chromatography on columns containing the appropriate APC fragment covalently coupled to activated tresyl-agarose (Schleicher and Schuell). Immunoprecipitations were performed by adding 1 to 3 μg of antibody to clarified extracts from 0.2 to 1 × 10^7 cells lysed in 1 ml of Triton X-100 lysis buffer [20 mM tris-HCl (pH 8.0), 1.0% Triton X-100, 140 mM NaCl, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM sodium vanadate, 50 mM NaF, 1 mM Pefabloc, and 10 μg/ml each of Aprotinin, pepstatin, and leupeptin]. After a 3-hour incubation at 4°C, protein A–Sepharose beads (20 μl; Sigma) were added and the mixture incubated for 1 hour. The beads were then washed three times with 1 ml each of buffer B [20 mM tris-HCl (pH 8.0), 150 mM NaCl, and 0.5% Nonidet P-40] or buffer B containing 1 M LiCl, and then eluted with SDS-PAGE sample buffer. For detection of APC on protein blots, affinity-purified antibodies were used at a final concentration of 0.2 μg/ml each. The antibody to β-catenin was raised against a synthetic peptide, and the antibody to E-cadherin against a fusion protein of glutathione-*S*-transferase and the cytoplasmic domain of mouse E-cadherin (*21*). Both antibodies were used at dilutions of 1:1000 for protein blots or 1:100 for immunoprecipitations. All SDS-PAGE was performed in 6% polyacrylamide gels, and protein blotting was performed with standard tris-glycine buffers containing 20% methanol. Protein blots were developed with horseradish peroxidase (HRP)–conjugated goat antibody to rabbit immunoglobulin G (Bio-Rad) and detection was by Enhanced Chemiluminescence (Amersham).
18. For "fishing" experiments, we used APC fragments containing the Glu-Glu epitope (consisting of the sequence Glu-Phe-Met-Pro-Met-Glu) at their NH_2-termini. The mAb to this epitope has been described (*22*). To identify radiolabeled associated proteins, we added 5 μg of the APC fragments to lysates prepared from half of the radiolabeled cells on a 10-cm dish. The mixture was incubated at 4°C for 3 hours and then immunoprecipitated with an antibody to Glu-Glu immobilized on protein G–Sepharose. For metabolic labeling, cells were starved for methionine for 2 hours and then incubated for 4 hours with [^{35}S]methionine (0.3 mCi per 10-cm dish). For purification of microgram amounts of the 95-kD protein, 10 T-150 flasks of confluent SW480 cells were extracted with 1% Triton X-100 lysis buffer and the extract clarified by centrifugation. Purified APC2 protein (200 μg), immobilized on anti-Glu-Glu-protein G–Sepharose, was incubated with the extract for 3 hours at 4°C and the beads were then

washed. The bound protein was eluted and subjected to SDS-PAGE as described for immunoprecipitations. The 95-kD protein was excised and equilibrated in 0.1M tris-HCl (pH 6.8), 0.1% SDS, 5 mM DTT, and 10% glycerol. The gel slices were inserted into the lanes of a 16% Tricine SDS-polyacrylamide gel, overlaid with 25 μl of Glu-C protease solution (20 μg/ml), and subjected to electrophoresis. The resolved fragments were electroblotted to PVDF membranes, and two fragments of ~6 to 8 kD were excised from the stained blot and sequenced.

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Association of the APC Tumor Suppressor Protein with Catenins

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Mutations of *APC* appear to initiate sporadic and inherited forms of human colorectal cancer. Although these mutations have been well characterized, little is known about the function of the *APC* gene product. Two cellular proteins that associate with APC were identified by nucleotide sequence analysis and peptide mapping as the E-cadherin–associated proteins α- and β-catenin. A 27-residue fragment of APC containing a 15–amino acid repeat was sufficient for the interaction with the catenins. These results suggest an important link between tumor initiation and cell adhesion.

Mutations in the *APC* tumor suppressor gene lead to benign colorectal tumors in about one-third of the population of the Western world (*1*). If not surgically removed, 15 to 20% of these tumors will progress to cancer. Understanding the mechanism by which APC mutations lead to tumorigenesis therefore has substantial practical, as well as theoretical, importance.

The *APC* gene was originally identified through positional cloning approaches and encodes a protein of 2843 amino acids (*2*). Somatic mutations in *APC* appear to initiate colorectal tumor development in the general population, whereas germline mutations result in familial adenomatous polyposis (FAP) (*3–5*), an autosomal dominantly inherited disease that predisposes individuals to colorectal cancer. Mice with germline mutations of APC manifest a phenotype similar to that of FAP (*6*). To date, more than 120 different germline and somatic mutations of APC have been identified, almost all of which result in COOH-terminal truncation of the gene product (*3–5*).

Although APC is known to form homooligomers (*7*), its predicted amino acid sequence provides few clues to its mechanism of action. To help elucidate the function of APC, we used immunoprecipitation to identify cellular proteins that interact with APC in vivo. We studied two human colorectal cancer cell lines—SW480 cells, which express an APC protein that is truncated at codon 1338 (*3*), and HCT116 cells, which express an apparently full-length APC protein (*7*). A monoclonal antibody (mAb) specific for the NH_2-terminus of APC (CF11) immunoprecipitates APC from both SW480 and HCT116 cells, whereas a mAb specific for the COOH-terminus (DB1) immunoprecipitates APC only from HCT116 cells (*7*). Proteins associated with APC should therefore be immunoprecipitated from HCT116 cells by both CF11 and DB1, but should not be immunoprecipitated from SW480 cells by DB1.

We found that two proteins of 95 kD (p95) and 100 kD (p100) were readily detectable in the immunoprecipitates when SW480 cell lysates were treated with CF11. In contrast, neither protein was detectable in the DB1 immunoprecipitates. When HCT116 cell lysates were used, p95 and p100 were immunoprecipitated by both antibodies (Fig. 1A) (*8*). These results suggested that p95 and p100 associate with APC and that the first 1337 amino acids of APC are sufficient for this interaction. Presumably because of its slow turnover rate, APC itself was not detectable in the immunoprecipitates by fluorography, but could be detected by immunoblotting (*7*).

To map the regions of APC that interact with p95 and p100, we examined the ability of different APC fragments to bind to these proteins in vitro. The APC fragments (Fig. 1C) were expressed in *Escherichia coli* as glutathione-S-transferase (GST) fusion proteins and tested for their ability to bind p95 and p100 from metabolically labeled SW480 cells (*9*, *10*). Only GST-APC(B), (G), and (H) bound to p95 and p100, indicating that the binding domain was

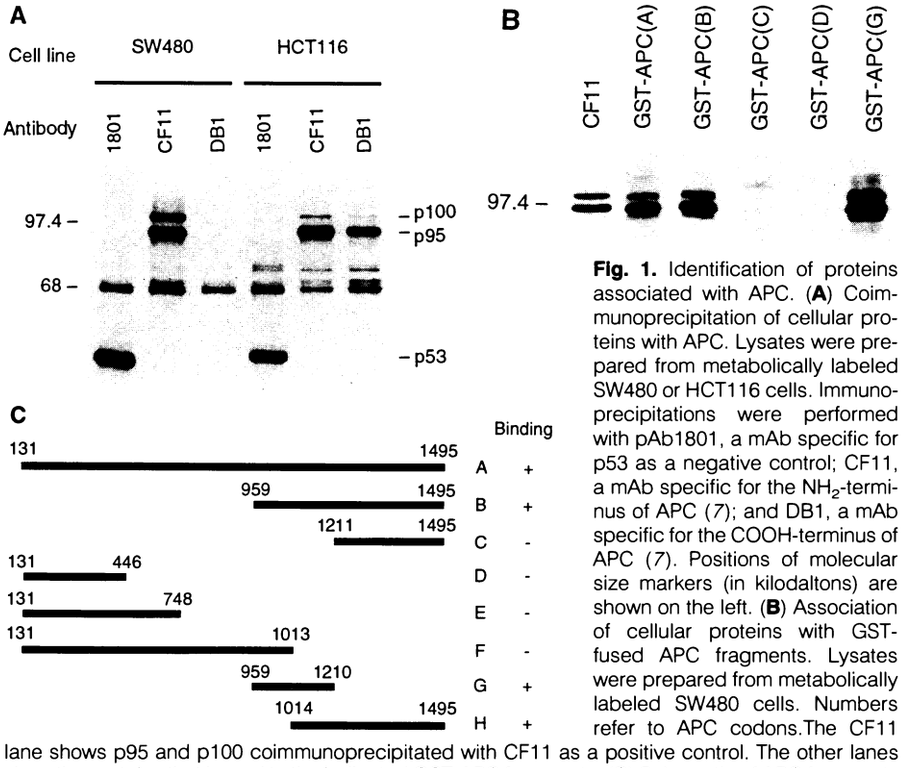

Fig. 1. Identification of proteins associated with APC. (**A**) Coimmunoprecipitation of cellular proteins with APC. Lysates were prepared from metabolically labeled SW480 or HCT116 cells. Immunoprecipitations were performed with pAb1801, a mAb specific for p53 as a negative control; CF11, a mAb specific for the NH_2-terminus of APC (*7*); and DB1, a mAb specific for the COOH-terminus of APC (*7*). Positions of molecular size markers (in kilodaltons) are shown on the left. (**B**) Association of cellular proteins with GST-fused APC fragments. Lysates were prepared from metabolically labeled SW480 cells. Numbers refer to APC codons.The CF11 lane shows p95 and p100 coimmunoprecipitated with CF11 as a positive control. The other lanes show the proteins that bound to the indicated GST-APC fragments. (**C**) Schematic of APC fragments expressed in bacteria as GST fusion proteins and summary of their ability to bind p95 and p100.

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