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Upstream Stimulating Factor-1 (USF1) and USF2 Bind to and Activate the Promoter of the *Adenomatous Polyposis Coli* (*APC*) Tumor Suppressor Gene

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Abstract The *adenomatous polyposis coli* (*APC*) gene product is involved in cell cycle arrest and apoptosis, and loss of function is associated with the development of colorectal carcinogenesis. Although it has been demonstrated that the *APC* gene is inducible, its transcriptional regulation has not been elucidated. Therefore, we characterized the promoter region of the *APC* gene and transcription factors required for basal expression. The *APC* gene has a TATA-less promoter and contains consensus binding sites for Octamer, AP2, Sp1, a CAAT-box, and three nucleotide sequences for E-box A, B, and M. The E-boxes are functional in several cancer cell lines and upstream stimulating factor-1 (USF1) and USF2 interact with these sites, with a preferred sequence-specificity for the B site. Analysis of activation of the cloned *APC* promoter by USF1 and USF2 in transient transfection assays in HCT-116 cells demonstrated that mutation of the E-box B site completely abolished the basal promoter activity. Further, the ectopic USF1 and USF2 expression in HCT-116 cells with deletion mutations of E-box A, B, and M sites showed that these E-boxes contribute to USF1- and USF2-mediated transcriptional activation of the *APC* promoter, with maximum promoter activity being associated with the E-box B site. Thus, USF1 and USF2 transcription factors are critical for *APC* gene expression. J. Cell. Biochem. 81:262–277, 2001. © 2001 Wiley-Liss, Inc.

Key words: *APC* gene; transcriptional regulation; E-box binding sites; USF1; USF2

Colon cancer is a major health problem, representing one of the most common causes of death from cancer in both men and women in the United States [Landis et al., 1998]. In the past few years, it has become clear that colon cancer stems from a series of somatic, genetic alterations and mutations in the adenomatous polyposis coli (*APC*) gene, which are thought to be one of the earliest events during this multi-step process of carcinogenesis [Ichii et al., 1992; Miyoshi et al., 1992; Powell et al., 1992; Taso and Shibata, 1994]. A direct causal relationship has been established between loss of function of the *APC* gene and the development of colorectal cancer in mice that carry a targeted conditional mutation of *APC* [Ahmed et al., 1998].

The *APC* gene product is a homodimeric protein, which is located both in the cytoplasm and in the nucleus [Smith et al., 1993; Miyashiro et al., 1995; Wong et al., 1996; Neufeld and White, 1997]. Its functional expression has been associated with cell cycle arrest [Baeg et al., 1995] and apoptosis [Browne et al., 1994; Morin et al., 1996]. In recent studies, the *APC* gene product has been described as a negative regulator of β-catenin signaling in several colon cancer [Rubinfeld et al., 1993; Su et al., 1993] and melanoma [Rubinfeld et al., 1996] cell lines. In the cytoplasm, *APC* forms a multimeric complex with β-catenin and glycogen synthase kinase-3β (GSK3β) that is assembled

Abbreviations used: APC, adenomatous polyposis coli; β-gal, β-galactosidase; bHLH, basic-helix-loop-helix; CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; pAPCP, adenomatous polyposis coli gene promoter; SDS, sodium dodecylsulfate.

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on an axin (also called conductin) backbone [Behrens et al., 1998; Hart et al., 1998; Nakamura et al., 1998]. Within this multimeric complex, GSK3β phosphorylates β-catenin resulting in dissociation of β-catenin from the complex and its subsequent degradation in the cytoplasm. If *APC* is absent or mutated, the binding of β-catenin to *APC* is impaired resulting in an increase in the cytoplasmic levels of β-catenin. The free cytoplasmic β-catenin is translocated to the nucleus, where it binds to members of the T-cell factor/lymphoid-enhancer factor (Tcf/Lef) family of transcription factors and activates target genes. It was reported recently that the activity of the *c-myc* oncogene is influenced by the β-catenin/Tcf-4 complex [He et al., 1998]. A β-catenin/Tcf-4 binding site in the *c-myc* promoter has been identified, and it was shown that overexpression of *APC* in an *APC*-mutant cell line resulted in a reduction in the nuclear levels of β-catenin/Tcf-4 complexes, which was associated with a decrease in the expression of the *c-myc* oncogene [He et al., 1998]. Thus, the interaction of *APC* with β-catenin may regulate an intracellular signaling pathway that links the cytoplasm and the nucleus.

In recent studies, we have shown that the levels of *APC* protein and mRNA were increased upon exposure of HCT-116 cells to DNA-damaging agents in culture [Narayan and Jaiswal, 1997; Jaiswal and Narayan, 1998]. Also, changes in the *APC* protein levels during nerve growth factor (NGF)-induced neuronal differentiation were examined in pheochromocytoma (PC12) cells [Dobashi et al., 1996]. These studies indicated that *APC* is an inducible gene, which can be regulated in response to a variety of signals induced by hormones and DNA-damaging agents. Thus, transcriptional regulation may also contribute to susceptibility to carcinogenesis and, an understanding of regulation at this level may lead to the design of more effective chemopreventive drugs that block tumor cell progression.

In this study, to examine the *APC* gene regulation, the *APC* promoter was cloned from the genomic DNA of HCT-116 cells and the transcriptional regulatory *cis*-elements of the *APC* promoter were analyzed. Transcriptional regulatory elements were identified that are specific for binding of basic-helix-loop-helix (bHLH) transcription factors (for review, see Olson, 1990; Dang et al., 1992; Atchley and Fitch, 1997) including the E-box A, B, and M motifs. The E-box site contains the consensus hexanucleotide sequence CANNTG. The central two nucleotides (NN) of the three E-boxes are GC (box A), CG (box B), and TG (box M). Interestingly, the identity of these two central nucleotides of the E-box may be sufficient to determine how bHLH proteins regulate the activity of a target promoter. However, the mechanisms by which bHLH proteins distinguish between these sites are unclear. Recently, using a yeast genetic system, Dang et al. [1992] showed that a single arginine in the basic region of the bHLH protein, c-Myc, is sufficient to discriminate between related E-box sites. bHLH proteins regulate a wide variety of tissue-specific genes that are involved in neurogenesis, morphogenesis, myogenesis, sex determination, cell proliferation, and differentiation (for review, see Olson, 1990; Dang et al., 1992; Reisman and Rotter, 1993; Atchley and Fitch, 1997; Yasumoto et al., 1997; Aksan and Goding, 1998).

The *APC* promoter is unusual in that it includes each of the E-boxes A, B, and M, which suggests a high degree of complexity in its regulation. Thus, the present study was undertaken to examine the roles of the E-box sites in the regulation of the *APC* promoter. Our results show that upstream stimulating factor-1 (USF1) and USF2 may serve as important regulators of *APC* promoter activity by binding to the E-box sites of different cell types including colon cancer cells.

MATERIALS AND METHODS

Oligonucleotides

For use in electrophoretic mobility shift assay (EMSA) complimentary single-stranded oligonucleotides were annealed to produce double-stranded oligonucleotides with the indicated sequence: E-box A (−48 to −29), 5′-GAGAGAAG**CAGCTG**TGTAAT-3′; E-box B (+33 to +52), 5′-GTGGGCG**CACGTG**ACCGACA-3′; EboxBmut1 (+33 to +52), 5′-GTGGGCt**gAtGTG**ACCGACA-3′; EboxBmut2 (+33 to +52), 5′-GTGGGCG**CACGgG**ACCGACA-3′; E-box M (+44 to +63), 5′-TGACCGA**CATGTG**GCTGTAT-3′. Several other oligonucleotides were synthesized with *Xba*I and *Kpn*I restriction endonuclease sites on the 5′ and 3′ ends of the E-box sites, respectively, for cloning and gene expression studies. After annealing with the

complimentary oligonucleotides, the DNA was digested with *Xba*I and *Kpn*I restriction endonucleases and then subcloned into CAT-reporter plasmids. The cloned plasmids and their oligonucleotide sequences were: p(EboxA)P (−44 to +7), 5′-GAAG**CAGCTG**TGTAATCC-GCTGGATGCGGACCAGGGCGCTCCCCATT-CCCG-3′; p(EboxB+M)P (+33 to +105), 5′-GT-GGGCG**CACGTG**ACCGA**CATGTG**GCTGTA-TTGGTGCAGCCCGCCAGGGTGTCACTGGA-GACAGAATGGAGGT-3′; p(EboxB)P (+33 to +105), 5′-GTGGGCG**CACGTG**-ACCGA-**CAcGcG**GCTGTATTGGTGCAGCCCGCCAG-GGTGTCACTGGAGACAGAATGGAGGT-3′; p(EboxM)P (+33 to +105), 5′-GTGGGCG-**CACGgG**ACCGA**CATGTG**GCTGTATTGGT-GCAGCCCGCCAGGGTGTCACTGGAGACAG-AATGGAGGT-3′; p(Inr)P (+83 to +105), 5′-GTCACTGGAGACAGAATGGAGGT-3′. The bold letters of the oligonucleotide sequences indicate the E-boxes and transcriptional initiation (Inr) sites, and the lower case letters indicate the mutations in these sites. The underlined letters indicate the transcriptional initiation site of the cloned promoters.

Plasmid Constructs

The *APC* promoter, located in the genomic sequence surrounding the 5′-exon of the gene, was identified and isolated previously [Eibner et al., 1994; Thliveris et al., 1994; Hiltunen et al., 1997; Esteller et al., 2000; Tsuchiya et al., 2000] (see Fig. 1). The nucleotide sequence of the *APC* promoter has been submitted to the gene bank (accession No. U02509). We cloned a 920 bp region of the *APC* promoter from the genomic DNA (pAPC) of HCT-116 cells and joined it to a chloramphenicol acetyl transferase (CAT)-reporter gene (pAPCP). This 5′-region of the *APC* gene is the major *APC* promoter, denoted as promoter 1A [Horii et al., 1993] or promoter.3 [Thliveris et al., 1994; Hiltunen et al., 1997; Esteller et al., 2000; Tsuchiya et al., 2000], from which the major *APC* transcript is initiated. Sequential unidirectional deletion of the pAPCP plasmid was performed to localize the *APC* core promoter and to assess the roles of different regulatory elements in positive or negative regulation of basal (uninduced) or induced *APC* gene expression. A site-specific mutation at +44 nt (T → G) was introduced within the E-box B site of the pAPCP promoter. This construct retains all other *cis*-elements with similar base pair spacing to the wild-type *APC* promoter, but lacks a functional E-box B site. This plasmid was named pAPCP(EboxB-mut2). The proper orientation of the insert was analyzed by DNA sequencing and/or restriction endonuclease digestion of each DNA construct.

Cell Culture and CAT-Reporter Assays

The human colon cancer cell line, HCT-116, was purchased from the ATCC (Rockville, MD). Cells were grown in MaCoy's 5a medium supplemented with 10% fetal bovine serum and 100 μg/ml penicillin and streptomycin. After reaching 60% confluence, cells were co-transfected with pAPCP (1 μg/ml) and β-galactosidase (β-gal) overexpressing plasmid pCMV-β-gal (0.2 μg/ml) using the Lipofectamine reagent (7 μg/ml) as described by the manufacturer (GIBCO BRL, Gaithersburg, MD). When indicated, cells were also co-transfected with 0.5 μg/ml of pSVO (control), pSV-USF1, and pSV-USF2 overexpression plasmids. After 60 h of transfection, cell lysates were prepared for the CAT-reporter assay [Hodge et al., 1995]. The CAT activity of each assay was normalized to β-gal activity in order to correct for differences in transfection efficiency.

Determination of Transcription Initiation Site

To identify the transcription initiation site, primer extension analysis of endogenous *APC* mRNA was performed as described previously [Jones et al., 1985]. A 26-mer primer, corresponding to positions +195 to +220 (5′-TGTC-TTAAACCGATGGCCTTTCCTTG-3′) of the *APC* promoter region in the anti-sense orientation, was used for primer extension analysis. To determine the transcriptional start sites, the primer extended products were analyzed using the above primer to sequence a segment of the cloned genomic DNA by the chain termination method [Sanger et al., 1977]. To further identify the transcriptional initiation site of the cloned pAPCP promoter, an in vitro run-off transcription assay was performed as described previously [Narayan et al., 1994]. A 973 bp DNA fragment, including 920 bp of the promoter region, was isolated by digesting the pAPCP plasmid with *Xba*I (5′ end) and *Kpn*I (3′ end) restriction enzymes. The in vitro run-off assay was performed using the *Xba*I/*Kpn*I fragment and a nuclear extract of HCT-116 cells. The run-off product and DNA size markers were analyzed by electrophoresis in a 6% denaturing polyacrylamide gel containing 8 M urea.

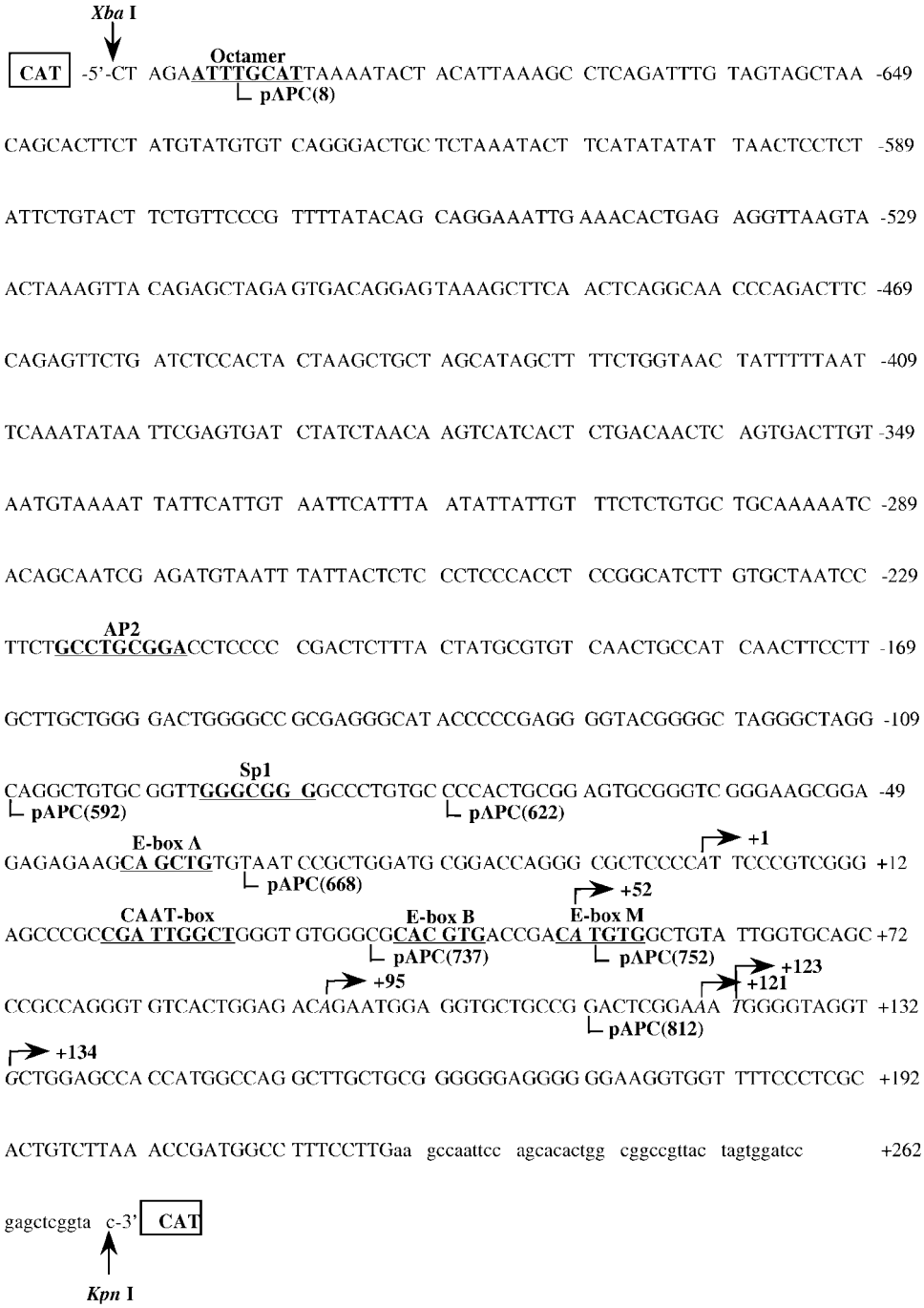

Fig. 1. Nucleotide sequence of the *APC* promoter region. The PCR-amplified promoter region of the *APC* gene was subcloned at the *Xba*I and *Kpn*I sites of the CAT-reporter gene. The putative *cis*-elements are shown in bold and underlined. Solid arrows indicate the transcriptional initiation sites of the gene and the initiating nucleotides are italicized. The functional *cis*-elements are underlined and written in bold letters. Endpoints of sequential deletion mutant plasmids are also shown.

Electrophoretic Mobility Shift Assays

The nuclear extracts were prepared as described previously for the electrophoretic mobility shift assay (EMSA) [Narayan and Jaiswal, 1997; Jaiswal and Narayan, 1998]. DNA–protein binding reactions were carried out in 20 μl containing 20 mM HEPES, pH 7.9; 1 mM DTT, 3.5 mM $MgCl_2$, 100 mM KCl, 0.03% (v/v) Nonidet P-40, 10% (v/v) glycerol, 1 μg poly (dI.dC), and 5 μg nuclear extract. Reactions were incubated at 22°C for 10 min, followed by

the addition of 1 ng [^{32}P]-labeled E-box oligonucleotide and incubation for 20 min. The entire reaction mixture was loaded directly onto a native 4% polyacrylamide gel. After electrophoresis, the DNA–protein complexes were visualized by autoradiography. For competition experiments, a molar excess of unlabeled oligonucleotide was added to the reaction mixture 10 min before the addition of [^{32}P]-labeled probe as indicated in the figure legends. For super-shift analysis, 1 μg each of USF1, USF2, c-*myc* (C-33), and Max (C-124) antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) were added to the reaction mixture and incubated for 20 min before the addition of [^{32}P]-labeled probe.

UV Cross-Linking

UV cross-linking was performed by a previously described procedure [He et al., 1996]. The reaction mixture was assembled as described above for EMSA. After 20 min incubation at 22°C to permit DNA–protein binding, the reaction mixture was spotted on parafilm and irradiated for 10 min at 254 nm using a UV-Stratalinker (Stratagene, La Jolla, CA). After boiling, the samples were analyzed by 9% SDS-PAGE. The gel was dried and exposed to X-ray film for identification of cross-linked DNA–protein complexes.

RESULTS

The Transcriptional Initiation Site of the pAPCP Promoter

The nucleotide sequence of the cloned *APC* promoter region (pAPCP) is shown in Figure 1. A 920 bp DNA fragment, amplified by PCR, containing 53 bp of the known pCR2.1 vector DNA sequence (Invitrogen, San Diego, CA) at the 3′ end was cloned into a CAT-reporter gene. The structure of the cloned promoter was determined by nucleotide sequence analysis, and was found to be identical to those published earlier except for a single nucleotide change at −171 (T → C) [Eibner et al., 1994; Thliveris et al., 1994]. It is likely that the transition from T → C residue was a result of PCR artifact, which does not seem to have an impact on the pAPCP promoter activity. To determine the transcriptional initiation sites, we first used primer extension analysis of the endogenous *APC* mRNA. The results shown in Figure 2A revealed several potential initiation sites in the *APC* promoter. Among these, we identified a consensus transcriptional initiation sequence (5′-CACTT-3′) located in the 5′ end of the *APC* promoter [Smale and Baltimore, 1989]. The 'A' residue of this transcriptional initiation motif was designated +1. Other transcriptional initiation sites were then designated as +52, +95, +121, +123, and +134 (Fig. 2A). Inspection of the sequences indicated that the cloned *APC* gene has a TATA-less promoter.

To determine whether the multiple transcriptional initiation sites of the *APC* promoter are functional in the cloned gene, we performed an in vitro run-off transcription assay using pAPCP promoter DNA and a nuclear extract of HCT-116 cells [Narayan et al., 1994]. Three major run-off transcripts were identified, which were initiating at the sites identified by primer extension analysis, i.e., +1, +52, and +95 (Fig. 2B, lane 1). One diffused faint band of run-off transcript can be seen (more visible after longer exposure of the gel) at the DNA molecular size of ~155 bp, which may be generated from the transcriptional initiation sites at +121 and +123 of the pAPCP promoter. Another faint band of the run-off transcript was present at the DNA molecular size of ~115 bp, which may be generated from the transcriptional initiation sites at +134 of the pAPCP promoter. These run-off transcripts were initiated by RNA polymerase II, because their production was sensitive to the presence of the specific inhibitor α-amanitin at a concentration of 1 μg/ml (Fig. 2B, lane 2).

Characterization of *APC* Promoter Activity Using a CAT-Reporter Assay

The object of this study is to elucidate the mechanisms by which the *APC* gene is transcriptionally regulated. Thus, it was necessary to identify the role of functional *cis*-regulatory elements in the *APC* promoter. The *APC* gene contains several different consensus *cis*-elements (Fig. 1), including potential binding sites for Oct-1, AP2, Sp1, and an element for a CAAT- box. In addition, there are three consensus sequences for E-box A, E-box B, and E-box M, which are binding sites for bHLH transcription factors (Fig. 1).

To examine the role of different *cis*-elements in the regulation of basal expression of *APC* gene, deletion mutants were generated in the promoter of the cloned pAPCP plasmid (Fig. 3A). These mutants were tested for

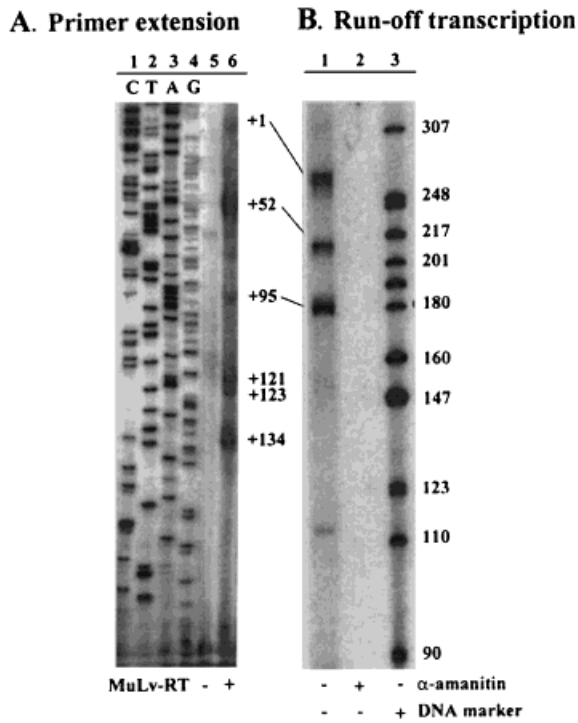

Fig. 2. Characterization of the pAPCP promoter transcriptional initiation site. **Panel A**: primer extension analysis. **Lanes 1–4**, chain termination sequencing reaction. **Lanes 5, 6**, a product from primer extension (without and with MuLV-RT, respectively) of total RNA of HCT-116 cells. The positions of the primer extended products are numbered starting from the extreme 5′ end product. **Panel B**: in vitro run-off transcription assay. The standard 25 μl transcription reaction contained 50 μg HCT-116 nuclear extract, 1 μg pAPCP plasmid, 5 mM $MgCl_2$, 65 mM KCl, 2 mM DTT, 10% (v/v) glycerol, and 20 units RNasin (Promega Inc., Madison, WI). The reaction mixture was incubated for 60 min to form preinitiation complexes. Transcription was initiated by adding ATP, UTP, GTP (500 μM), and 25 μM CTP with 7.5 μCi of [α-^{32}P]CTP (800 Ci/mmol; ICN Biochemicals, Inc., Costa Mesa, CA) for a 30 min incubation at 22°C. Reactions were stopped and run-off products were analyzed on a 6% acrylamide/8 M urea gel. The dried gel was exposed to X-ray film for autoradiography. **Lane 1**: run-off products matched with initiation sites of the primer extended products. **Lane 2**: run-off products in the presence of 1 μg/ml α-amanitin. **Lane 3**: DNA size markers.

promoter function in a CAT-reporter assay (Fig. 3B). The results show that Oct-1, AP2 and Sp1-binding sites, E-box and CAAT-box sites, and factors interacting with these sites may play an important role in the function of the pAPCP promoter. The Octamer site may act as an enhancer, because deletion of the Octamer site in mutant plasmid pAPCP(8) decreased promoter activity. The mutant plasmid pAPCP(592), which lacks the AP2-binding site and possibly other unknown *cis*-elements, displays increased promoter activity indicating that this region of 586 bp DNA may have repressor element(s). Deletion up to the E-box A site did not change pAPCP promoter activity, but further deletion that resulted in the removal of the CAAT-box site decreased promoter activity (Fig. 3B, compare CAT-activities of pAPCP(622), pAPCP(668) and pAPCP(737)). The results suggest that CAAT-box element is an activator site of the pAPCP promoter. Deletion up to the E-box B and E-box M sites reduced the level of pAPCP promoter activity to that observed on deletion up to the CAAT-box. Based on the promoter deletion studies, the results indicate that the region between the transcriptional start site and the CAAT-box included in plasmid pAPCP(668), is the core *APC* promoter which is responsible for basal-level activity.

However, from the promoter deletion analysis, it is unclear whether E-box sites play a role in the regulation of the *APC* gene expression. The pAPCP mutants generated by directional deletion do not reveal the specific effect of each *cis*-acting regulatory element on the promoter, because all of the constructs, except for pAPCP(8), resulted in deletion of more than one *cis*-acting element from the wild-type promoter. The plasmid that was deleted through the CAAT-box displayed a similar level of promoter function as the plasmid bearing the deletion that removed the E-box B and E-box M sites; thus, these constructs may not clearly illustrate the potential functions of the E-box B and E-box M sites. Therefore, site-directed mutation of the E-box B sequence was performed, so that the CAT-reporter assay system could be used to test the functional importance of the E-box B and E-box M elements. A single (T → G) base mutation was created at nt position +44 of the E-box B site, yielding the plasmid pAPCP(EboxBmut2). When this construct was transfected into HCT-116 cells, the CAT-reporter activity was 85% lower than that of the wild-type pAPCP (Fig. 3B, compare results of the first lane of the pAPCP with the last lane of the pAPCP(EboxBmut2) promoter). These results suggest that the E-box B site and the proteins binding to this site function as activators of the *APC* promoter in vivo, and that an intact E-box B site is required for full promoter activity. Furthermore, a single T residue at nt position +44 of pAPCP appears to be critical for E-box B-mediated promoter activity.

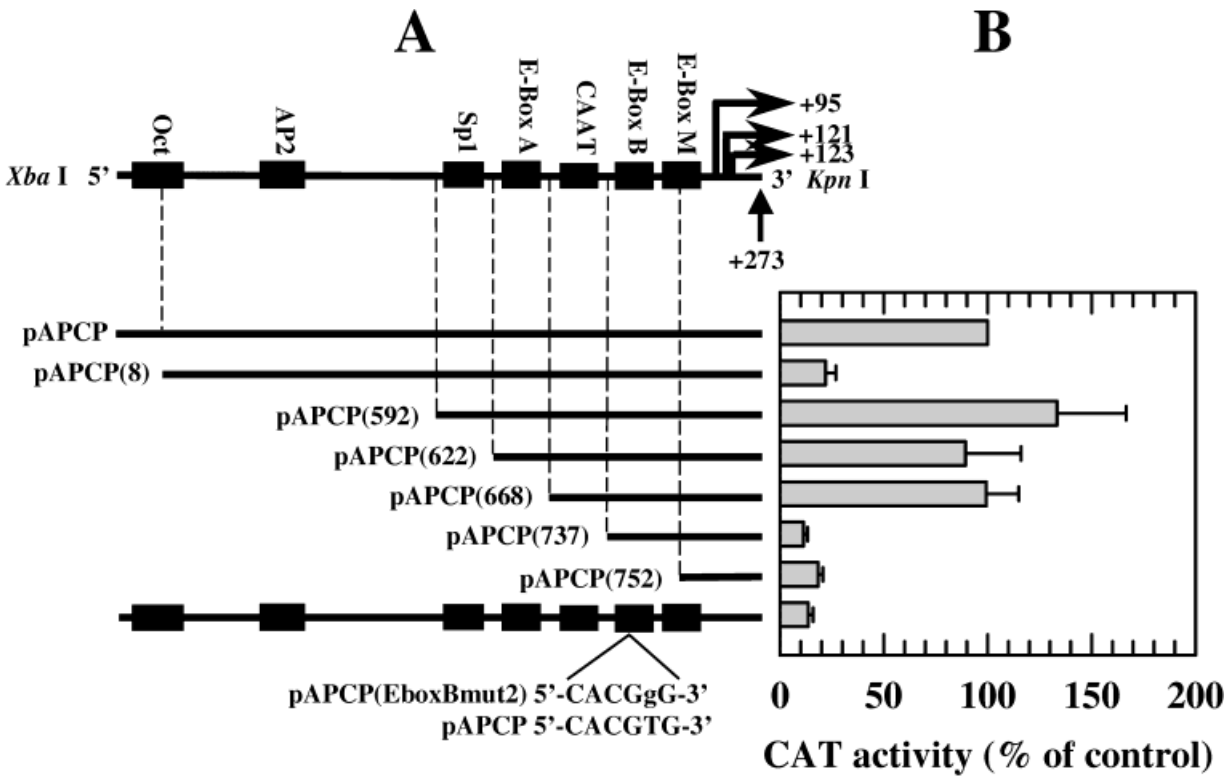

Fig. 3. Characterization of pAPCP basal promoter activity and identification of functional *cis*-elements by the CAT-reporter assay. **Panel A**: structure of the *APC* promoter-CAT construct and deletion mutants thereof. **Panel B**: CAT activity. HCT-116 cells were co-transfected with various deletion mutants using Lipofectamine Reagent. The CAT-reporter activity for each promoter construct is shown. Data were normalized to β-gal activity in the same experiment and are the mean ± SE of three different experiments.

Characterization of the E-box-Binding Sites of the pAPCP Promoter by Electrophoretic Mobility Shift Analysis

Electrophoretic mobility shift analyses (EMSA) were carried out to analyze the interactions and binding specificities of HCT-116 nuclear factors with the E-boxes of the pAPCP promoter. Double-stranded DNA oligonucleotide-binding substrates were constructed containing 20 nt segments of the pAPCP promoter. The substrates included sequences from either E-box A (−48 to −29 nt), E-box B (+33 to +52 nt), or E-box M (+44 to +63 nt) (see Materials and Methods). The proteins in the HCT-116 nuclear extract formed DNA–protein complexes with all of the E-box oligonucleotides, but with different binding affinities (Fig. 4A). The binding of the nuclear extract with the E-box A oligonucleotide substrate was very weak. The DNA–protein complex formed with the E-box B oligonucleotide was eliminated by competition with a 50-fold excess of unlabeled E-box B oligonucleotide (Fig. 4A, lane 7), but was unaffected by a 50-fold excess of unlabeled E-box A or E-box M oligonucleotides (Fig. 4A, lanes 6 and 8). The DNA–protein complex formed with the E-box M oligonucleotide was eliminated by competition with a 50-fold excess of unlabeled E-box B and E-box M oligonucleotides, but not with a similar concentration of unlabeled E-box A oligonucleotide (Fig. 4A, compare lane 9 with lanes 10–12). These results indicate that proteins of the HCT-116 nuclear extract interact and bind to the E-box B and E-box M sites, with the binding affinity for E-box B being much greater than that for E-box M. Thus, E-box B and M sites may play a major role, and E-box A, a minor role in the transcriptional regulation of the pAPCP promoter. Furthermore, the differences in the competition of unlabeled E-box oligonucleotides with cross-competing [^{32}P]-labeled E-boxes may be due to influence of neighboring nucleotides on

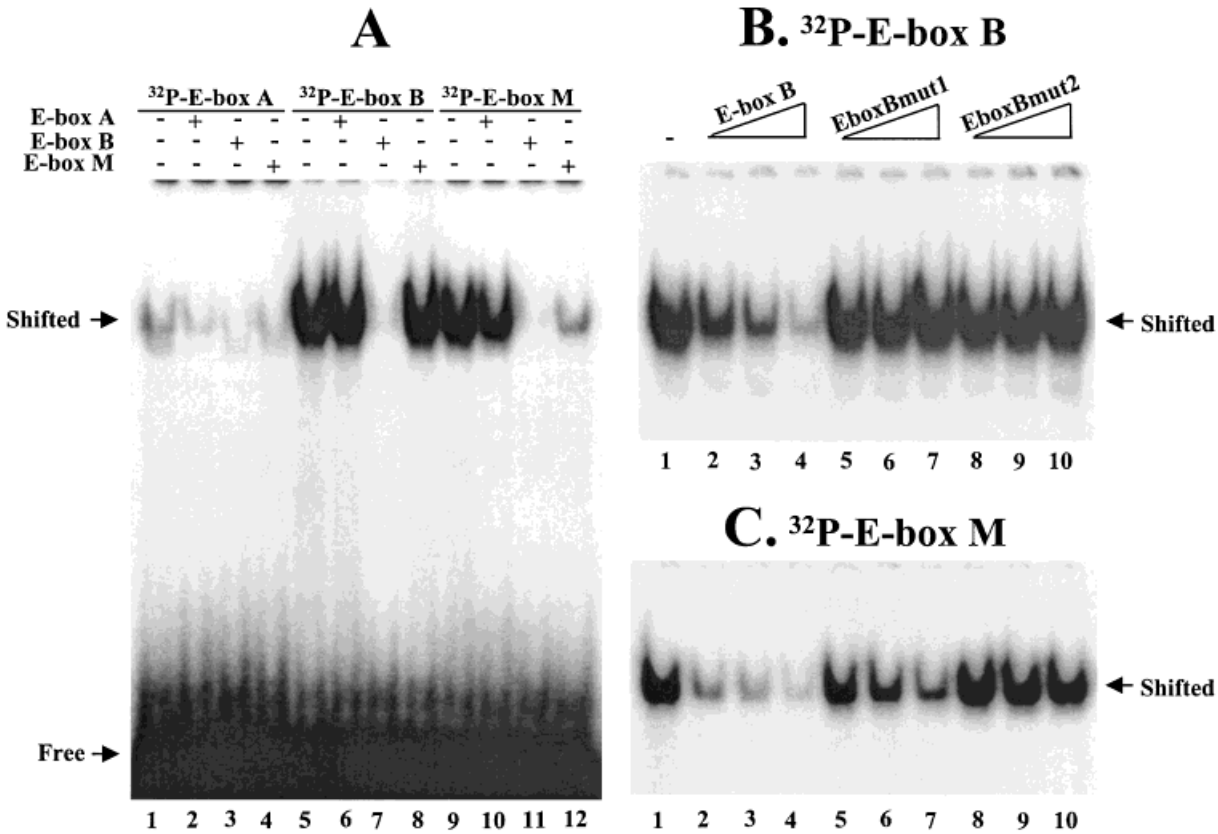

Fig. 4. Characterization of binding of E-box A, B, and M sites of the pAPCP promoter to HCT-116 nuclear extract. **Panel A**: binding of [^{32}P]-labeled E-box A, B, and M site oligonucleotides of the pAPCP promoter to the HCT-116 nuclear extract. The oligonucleotide sequences used in these studies are shown in Materials and Methods. After the binding reaction, the DNA–protein complexes were resolved on a 4% non-denaturing acrylamide gel. The [^{32}P]-labeled E-box A, B, and M site oligonucleotide probes were used as binding substrates. The positions of the shifted DNA–protein complex and the free probe are shown with arrows. A 50-fold excess of unlabeled competitor E-box-binding site oligonucleotide was present in the lanes where indicated. Sequence-specific binding of the HCT-116 nuclear extract with the E-box B site (**Panel B**) and the E-box M site oligonucleotides (**Panel C**) of the pAPCP promoter is shown. The fold excess of the competitor oligonucleotides were: E-box B, 0, 5, 10, 25; EboxBmut1 and EboxBmut2, 0, 25, 50, 100; Sp1 and CAAT-box, 0, 10, 25, 50. The shifted DNA–protein complex and the free probes are shown with arrows (Panel A). The autoradiograms of Panel B and C are a portion of the gel showing only the shifted bands of the DNA–protein complex.

the promoter region, which may determine binding affinity of E-box-binding proteins on the E-box sites.

We next analyzed the specificity of binding to E-box B and E-box M oligonucleotides in greater detail through the use of different concentrations of unlabeled wild-type and mutant oligonucleotide competitors (Fig. 4B, C). The DNA–protein complex with the [^{32}P]-labeled E-box B was eliminated with as little as a 25-fold excess of unlabeled wild-type E-box B oligonucleotide (lanes 2–4), but was unaffected even with a 100-fold excess of unlabeled EBoxBmut1 (lanes 5 and 6) or EBoxBmut2 (lanes 8–10) oligonucleotides (Fig. 4B). Similar to the E-box B site, the DNA–protein complex of the [^{32}P]-labeled E-box M oligonucleotide was competitively inhibited by a 50-fold excess of unlabeled wild-type E-box B oligonucleotide (Fig. 4C, lanes 1–4). Identical results were obtained when the competitor was unlabeled wild-type E-box M oligonucleotide (data not shown). The [^{32}P]-labeled E-box M oligonucleotide-binding also was competitively inhibited by a 50-fold excess of unlabeled EboxBmut1, but not with EboxBmut2 oligonucleotides. Taken together, these results indicate that the DNA–protein complex formed between the HCT-116 nuclear extract and the E-box B and E-box M oligonucleotides of the *APC* promoter is sequence-specific, and that the binding shows greater specificity for the E-box B site.

Identification of Nuclear Factors That Bind to the E-Box Sites of the pAPCP Promoter

The following experiments were carried out to determine the identity of the bHLH protein(s) that interact with the E-box sites of the *APC* promoter. In EMSA studies, one DNA–protein complex was detected as a single band that represents the complex formed between the [^{32}P]-labeled E-box A, B, and M oligonucleotides and HCT-116 nuclear extract proteins (Fig. 4). We used UV cross-linking of the DNA–protein complex(es) to facilitate identification of their protein components. UV cross-linking is carried out on the DNA–protein complex(es) in solution under non-denaturing conditions followed by separation of the cross-linked proteins by SDS-PAGE, such that the molecular size of the protein(s) can be determined. Consistent with the EMSA results, one strong DNA–protein band was observed after UV cross-linking with [^{32}P]-labeled E-box B and E-box M oligonucleotides. This diffused DNA–protein band had a molecular size in the range of 54–57 kDa. After subtracting the molecular mass of the E-box B or E-box M oligonucleotides (~13 kDa), the apparent molecular weight of the major protein in the DNA–protein complex was calculated to be in the range of ~42–44 kDa (Fig. 5A). Two minor bands of 75 and 137 kDa also were detected. The apparent molecular weight of these proteins (after subtracting the molecular mass of the oligonucleotide) matches those of c-Myc and initiator-binding protein (TFII-I), respectively. The specificity of the ~54–57 kDa DNA–protein complex for the E-box A, B, and M sites was determined by carrying out UV cross-linking in the presence of a 50-fold excess of unlabeled wild-type E-box A, B, and M oligonucleotides. Under these conditions the ~54–57 kDa DNA–protein complex in the presence of excess of unlabeled E-box B oligonucleotide was not detected. Furthermore, when a 50-fold

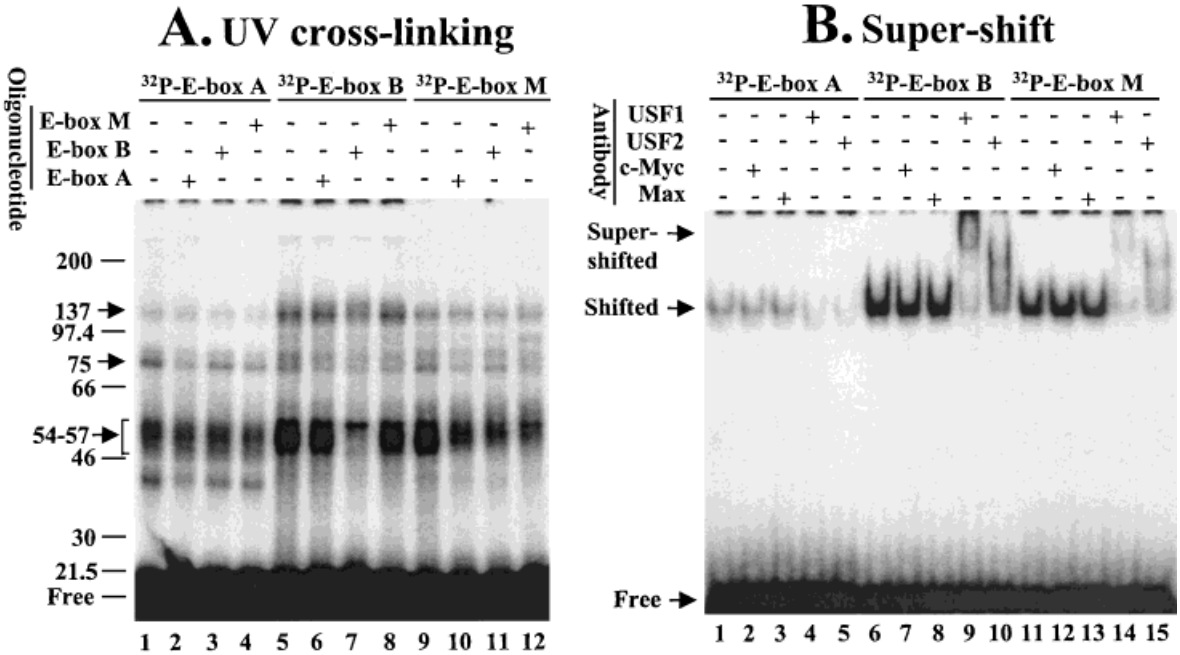

Fig. 5. Analysis of the binding of [^{32}P]-labeled E-Box B site oligonucleotide to USF1 and USF2 in HCT-116 nuclear extract. **Panel A**: UV cross-linking of the [^{32}P]-labeled E-box A, B, and M site oligonucleotidse to HCT-116 nuclear extract. The [^{32}P]-labeled E-box site oligonucleotides were incubated with HCT-116 nuclear extract under non-denaturing conditions and then exposed to UV radiation as described in Materials and Methods. The cross-linked DNA–protein complexes were analyzed by SDS-PAGE under denaturing conditions and detected by autoradiography. The approximate molecular sizes of the proteins of these complexes (shown in a parenthesis) were determined after subtracting the molecular mass (~13 kDa) of the [^{32}P]-labeled E-box site oligonucleotides. Where indicated, a 50-fold excess of the competitor oligonucleotide was included in the incubation mixture. **Panel B**: USF1 and USF2 bind to pAPC-CAT promoter as a heterodimer. The DNA–protein complex of shifted and antibody super-shifted bands were resolved on a 4% non-denaturing acrylamide/bis-acrylamide gel. The shifted and super-shifted DNA–protein complex bands and free probes are shown with arrows. Antibodies were included in binding assays as indicated.

excess of the E-box A or E-box M oligonucleotides was used as an unlabeled competitor, the formation of the ~54–57 kDa DNA–protein complex was unaffected (Fig. 5A, lanes 6 and 8). The ~54–57 kDa DNA–protein complex of the [^{32}P]-labeled E-box M oligonucleotide, on the other hand, was competed with a 50-fold excess of unlabeled E-box A, B, and M oligonucleotides (Fig. 5A, lanes 9–12). Taken together, these results indicate that the ~54–57 kDa DNA–protein complex is specific for the E-box B site but, a similar protein(s) could also bind to the E-box M site of the pAPCP promoter.

Since ~54–57 kDa DNA–protein band was diffused, it might consist of one or more similarly sized DNA–protein complexes. The apparent molecular masses of bHLH proteins USF1 and USF2 are 43 and 44 kDa, respectively. These proteins bind to E-box sites of their target gene promoters [Sawadogo, 1988; Sirito et al., 1994] suggesting that USF1 and USF2 are candidates to complex with E-box sites of the pAPCP promoter. We performed EMSA in the presence of antibodies against USF1 and USF2 to test this possibility. Since the bHLH proteins, c-Myc and Max, bind to the E-box site, c-Myc and Max antibodies were also included in super-shift assays. Figure 5B shows that the DNA–protein complex of the [^{32}P]-labeled E-box A, B, and M was super-shifted only by antibodies to USF1 and USF2 (lanes 4 and 5, 9 and 10, 14 and 15, respectively), and was unaffected by antibodies to c-Myc and Max (lanes 2 and 3, 7 and 8, 12 and 13, respectively). These results suggest that a heterodimer of USF1 and USF2 may bind to the E-box sites and act to activate the pAPCP promoter.

USF1 and USF2-Dependent Activation of the pAPCP Promoter After Co-Transfection Into HCT-116 Cells

The results presented in Figure 5 demonstrate that USF1 and USF2 bind specifically at the E-box sites of the pAPCP promoter and so, suggest that USF1 and USF2 may have functional roles in the regulation of the pAPCP activity. To test this hypothesis, HCT-116 cells were co-transfected with either empty vector (pSVO), or with USF1 (pSV-USF1), or with USF2 (pSV-USF2) expression constructs, along with CAT-reporter plasmids pAPCP (wild-type), pAPCP(737) (containing E-box B and E-box M sites, and two major transcriptional initiation sites around +52 and +95 nt), pAPCP(752) (containing only the transcriptional initiation site around +95 nt), and the pAPCP(812) plasmid which is devoid of +1, +52, and +95 transcriptional initiation sites. The cells also were co-transfected with a β-gal expression plasmid to normalize CAT-activity. Our results show that the ectopic expression of USF1 and USF2 significantly stimulated the reporter activity of pAPCP (wild-type), pAPCP(737), and pAPCP(752) promoters. The CAT-reporter activity of the pAPCP promoter (3–4-fold), pAPCP(737) promoter (9–11-fold), and pAPCP(752) promoter (~2-fold) were enhanced (Fig. 6A). These results indicate that binding of USF1 and USF2 to E-box B and E-box M sites may enhance pAPCP(737) promoter activity (Fig. 6A). Furthermore, ~2-fold increased CAT-reporter activity of the pAPCP(752) promoter (which does not contain E-box B or M sites) and the pAPCP(812) promoter (which lacks the major transcriptional initiation sites +1, +52, and +95), after ectopic USF1 or USF2 expression, appeared to be independent of the presence of the transcriptional initiation site. These results suggest that ~2-fold increase in the CAT-activity of pAPCP(752) and pAPCP(812) promoters might be due to transfected DNA, instead of the over expression of USF1 and USF2 proteins. Thus, the transcriptional initiation sites of the pAPCP promoter may not affect USF1- and USF2-mediated regulation of the *APC* gene expression.

Examination of CAT-reporter activity in HCT-116 cells co-transfected with both pAPCP(EboxBmut2) and pSV-USF1 or pSV-USF2 overexpression vectors resulted in a 12-fold increase in the pAPCP(EboxBmut2) promoter activity in the presence of ectopic USF1 or USF2 (Fig. 6A). Since the pAPCP(EboxBmut2) plasmid contains functional E-box A and E-box M-binding sites, it is likely that USF1 and USF2 interact with and stimulate pAPCP (EboxBmut2) promoter activity through these sites. However, the mechanism by which USF1 and USF2 regulate the promoter activity of these CAT-reporter constructs is not readily apparent. Furthermore, the nature of the interaction of USF1 and USF2 with the different E-box sites and the function of these transcription factors cannot be examined using this approach in which other *cis*-regulatory elements of the pAPCP promoter are present.

To determine whether the E-box sites of the pAPCP promoter function independently or

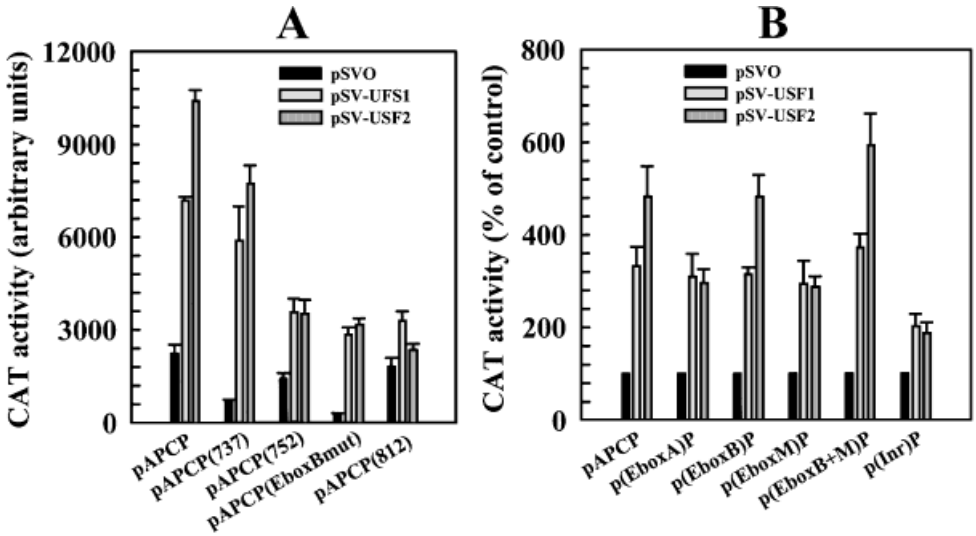

Fig. 6. Transcriptional activation of the cloned *APC* promoter after ectopic USF1 and USF2 expression in HCT-116 cells. **Panel A**: HCT-116 cells were co-transfected with pAPCP, pAPCP(737), pAPCP(752), pAPCP(812), and pAPCP(EboxB-mut2) promoters with pSVO (control), pSV-USF1, and pSV-USF2 overexpression vectors as indicated. The CAT-reporter activity was normalized to β-gal activity. Data are mean ± SE of three different experiments. **Panel B**: E-box A, B, and M sites independently can stimulate USF1- and USF2-mediated CAT-reporter activity. To study the transcriptional regulation of pAPCP by E-boxes, different oligonucleotides containing E-box A, B, B+M, and M were synthesized and cloned into a CAT-reporter plasmid. These plasmids were co-transfected with pSVO, pSV-USF1, or pSV-USF2 vectors into HCT-116 cells and the CAT-reporter activity was determined. After normalization with β-gal activity, the data of mean ± SE of three independent experiments is shown.

whether the presence of all three E-boxes is required, and to further determine whether transcriptional initiation sites can respond to USF1 or USF2 in the absence of the E-box sites, synthetic oligonucleotides corresponding to different E-boxes and transcriptional initiation sites were cloned into a CAT-reporter plasmid. Each reporter construct was then co-transfected into HCT-116 cell line with pSVO (control), pSV-USF1, or pSV-USF2 overexpression vectors. Results showed that ectopic expression of both USF1 and USF2 into the HCT-116 cell line stimulated the activity of the p(EboxA)P, p(EboxB)P, and p(EboxM)P promoters (Fig. 6B). As revealed by the EMSA studies described above, both USF1 and USF2 have strong binding affinity for the E-box B site (Figs. 4, 5), and a similar effect was found on promoter activity. Activation of the p(EboxB)P promoter, but not the p(EboxA)P and p(EboxM)P promoters, by USF1 or USF2 was comparable to that of pAPCP (wild-type) promoter (Fig. 6B). The activity of the p(EboxB+M)P promoter was higher than pAPCP or p(EboxB)P promoters after co-transfection with USF1 or USF2. These results indicate that all three E-boxes can regulate the *APC* promoter activity independently, but with a varying degree of specificity. The p(Inr)P plasmid showed less than 2-fold activation of the CAT-reporter activity after ectopic expression of USF1 or USF2, further suggesting that E-box sites rather than the transcriptional initiation site are important for USF1- and USF2-mediated activation of the pAPCP promoter. The p(Inr)P plasmid contains the nucleotide sequences of the transcriptional initiation site at +95. However, from these studies, it is not clear whether other transcriptional initiation sites play a role in the USF1- and USF2-mediated transcriptional regulation of the pAPCP promoter.

DISCUSSION

In order to understand the mechanism(s) underlying the transcriptional regulation of the *APC* gene, it is critical that the functional *cis*-regulatory elements in the gene's promoter region be identified. In previous studies, the 5′-end of the genomic DNA containing the *APC* promoter region was described [Eibner et al., 1994; Thliveris et al., 1994; Hiltunen et al., 1997; Esteller et al., 2000; Tsuchiya et al., 2000]. In the present work, the *APC* promoter

region was cloned from the genomic DNA of HCT-116 cells and studies were carried out to elucidate the mechanisms of *APC* gene regulation. The results are consistent with previous studies of *APC* promoter 1A [Horii et al., 1993] or *APC* promoter.3 [Eibner et al., 1994; Thliveris et al., 1994; Hiltunen et al., 1997; Esteller et al., 2000; Tsuchiya et al., 2000] and indicate that the mammalian *APC* gene has a TATA-less promoter. Multiple transcriptional initiation sites at +1, +52, +95, +121, +123, and +134 residues were observed for the *APC* promoter, which is a common feature of many TATA-less promoters [Widen et al., 1988; Tatsuka et al., 1995; Suzuki et al., 1998], but their functional significance in the *APC* promoter is unclear. The *APC* promoter contains potential binding sites for Oct-1, AP2, and Sp1. Besides these, the *APC* promoter also contains three consensus sequences for E-box A, B, and M, and one for the CAAT-box. The results presented here suggest that the DNA sequences for Octamer, AP2, Sp1, E-box B, and CAAT-box sites may be important for the regulation of *APC* promoter's basal activity in vivo. These *cis*-elements and the factors interacting with these sites may also play an important role in the activation of the *APC* promoter.

Although, Octamer and CAAT-box binding sites of the *APC* promoter are important for the regulation of *APC* gene expression, in this study we focused on characterizing the functional role of the three E-box consensus sites in the *APC* promoter. We identified three E-boxes on the cloned pAPCP promoter. Several reports have indicated that many bHLH transcription factors binding to E-boxes act as regulators of a large number of genes. The bHLH factors of the MyoD family, which regulate muscle-specific structural genes, bind to the E-box A site of their target promoters [Blackwell and Weintraub, 1990; for review, see Olson, 1993]. Other bHLH proteins regulate promoters containing E-box B sites [Atchley and Fitch, 1997]. These proteins include USF1/USF2 [Luo and Sawadogo, 1996; Sirito et al., 1998], Myc/Max [Blackwell et al., 1990; Blackwood and Eisenman, 1991], Mad/Max [Ayer et al., 1993], Max/Myn [Ayer et al., 1993], Mad/Mxi1 [Zervos et al., 1993], and Max/Mxi1 [Schreiber-Agus et al., 1995]. Another class of bHLH proteins binds to E-box M sites in a tissue-specific manner and regulates tyrosinase gene family promoters [Yasumoto et al., 1997; Akson and Goding, 1998]. In the present studies, we examined the binding specificity of HCT-116 nuclear extract with different E-box sites of the *APC* promoter. Binding studies using electrophoretic gel-mobility shift assays demonstrated that the E-box A site oligonucleotide formed only a weak DNA–protein complex with the HCT-116 nuclear extract. In contrast, the E-box B and M site oligonucleotides showed specific binding with the HCT-116 nuclear extract, indicating that the E-box B and M sites of the pAPCP promoter are important for the regulation of *APC* gene expression (Fig. 4). It was also demonstrated by UV cross-linking and antibody super-shift analyses that USF1 and USF2 bHLH proteins bind with higher affinity to the E-box B and M sites of the pAPCP promoter, although the binding of the E-box B site was found to be more specific than the binding of the E-box M (Fig. 5). These results suggest that factors binding to these sites are critical in regulating *APC* gene expression.

As discussed above, several well-known bHLH transcription factors bind to E-box sites of their target promoters, including USF1, USF2, c-Myc, Max, Mad, Myn, and Mxi-1. These were shown to bind to and regulate the activity of target promoters containing E-box B sites [Blackwell et al., 1990; Blackwood and Eisenman, 1991; Prendergast et al., 1991; Ayer et al., 1993; Zervos et al., 1993; Schreiber-Agus et al., 1995; Luo and Sawadogo, 1996; Sirito et al. 1998]. The transcription factor c-Myc is known to form a heterodimeric complex with Max, and the binding of this complex to an E-box B site results in the transactivation of target genes that include *p53* [Reisman and Rotter, 1993], *ornithine decarboxylase* [Bello-Fernandez et al., 1993; Pena et al., 1995], *cdc25A* [Galaktionov et al., 1996], and *α-prothymosine* [Gaubatz et al., 1994]. The USF1 and USF2 transcription factors are expressed ubiquitously and form homodimers or heterodimers that stimulate the transcriptional activity of promoters [Sirito et al., 1994]. Unlike the Myc family of proteins, the USF1 and USF2 proteins have not been reported to heterodimerize with other bHLH proteins. In the present studies, we found that both USF1 and USF2 can bind and activate the pAPCP promoter in transient transfection assays. The selective interaction of these factors with the consensus DNA-binding site may depend upon

the flanking nucleotide sequences and/or tissue-specificity, because different factors may be involved in competition for the same binding site. In addition, transcription factors binding adjacent to the E-box B and M sites can influence the binding specificity of these factors [Bendall and Molloy, 1994; Genetta et al., 1994; Weintraub et al., 1994; Desbarats et al., 1996]. However, currently it is not known how USF1 and USF2 selectively discriminate and bind to the pAPCP promoter, especially when c-Myc and other bHLH proteins apparently do not. In previous studies, discrimination among the family members that bind to the E-box sites of certain genes has been described [Blackwell et al., 1990]. Recently, an alternative model has been suggested in which the specific binding of bHLH proteins (by, for example, the c-Myc target genes) was shown to be determined through discrimination at the level of post-DNA binding mechanisms [Boyd et al., 1998].

The mutational studies described here indicate that the E-box B site of the *APC* promoter is important for basal level gene expression. Overexpression of USF1 or USF2 in HCT-116 cells stimulated promoter activity of the wild-type pAPCP (2.5-fold), as well as of pAPCP(737) (9–11-fold), which contains only the E-box B and M sites (Fig. 6A). These results suggest that the lower wild-type promoter activity of the pAPCP induced by USF1 and USF2 may be due to interference by other transcription factors such as those binding to the Octamer, ISRE, AP2, Sp1, and CAAT-box sites. It is not known which other transcription factors may be interacting with USF1 and USF2 during assembly of the transcriptional preinitiation complex at the *APC* promoter. Similar results were obtained when artificially cloned E-box sites were examined for USF1- or USF2-mediated CAT-reporter activity (Fig. 6B). Taken together, our results suggest that USF1 and USF2 might be key regulators of *APC* gene expression.

Site-directed mutagenesis of the E-box B site allowed precise analysis of the role of E-box B in *APC* promoter function. Our results obtained with the E-box B-mutant promoter, pAPCP(EboxBmut2) suggest that USF1 and USF2 can activate the *APC* promoter independent of the E-box B site, perhaps through involvement of the E-box A and M sites. However, the mechanism by which USF1 and USF2 may be regulating the *APC* gene expression through the E-box sites is currently unknown. USF1 and USF2 can function independently or interact with other transcription factors to form transcriptional preinitiation complexes. In fact, previous studies described interactions between USF1 and an initiator-binding protein TFII-I [Roy et al., 1991; Du et al., 1993; Li et al., 1994; Roy et al., 1997]. USF1 has also been shown to enter into the transcriptional preinitiation complex through binding with the $TAF_{II}55$ subunit of the TFIID complex [Chiang and Roeder, 1995]. The TFIID complex can be recruited onto a TATA-less promoter through interaction with the initiator-binding protein TFII-I [Roy et al., 1993]. Thus, it is possible that USF1 and USF2 may interact with the TATA-less promoter of *APC* through the TFIID/TFII-I complex. However, it cannot be ruled out that USF1 or USF2 interact with transcription factors other than transcriptional initiation site-binding factors, such as those binding to Octamer, ISRE, AP2, Sp1, or CAAT-box sites. Although the significance of the interaction of USF1 and USF2 with different transcription factors in regulating *APC* gene expression is not clear, the activation of the pAPCP promoter by USF1 and USF2 may require a transcriptional initiation site. These results indicate that the interaction of different transcription factors with the transcriptional initiation site may establish a stable and transcriptionally active preinitiation complex. Alternatively, USF1 and USF2 may form multimeric complexes with transcriptional initiation site-binding proteins, occupy E-box B and M sites along with an Inr-binding site, and may activate the *APC* promoter. For example, expression of CAT-reporter activity was induced with pAPCP or pAPCP(737) promoters after ectopic expression of USF1 or USF2, which contained functional E-box B and M sites and the transcriptional initiation site. Furthermore, even in the absence of the E-box B site, USF1 and USF2 may form multimeric complexes with E-box M and transcriptional initiation site-binding protein(s) and other transcription factors to stimulate the *APC* promoter activity, e.g., the stimulation of promoter activity of the pAPCP(EboxBmut2) plasmid by ectopic USF1 and USF2 (Fig. 6). A similar phenomenon has been suggested for USF-dependent regulation of the adenovirus major late promoter [Du et al., 1993; Li et al., 1994; Roy et al., 1997].

USF1 and USF2 can bind to and activate their target promoters by binding to an E-box site as homo or heterodimers and the cytoplasmic levels may vary among different cell types [Sirito et al., 1994]. In HCT-116 cells, the level of USF1 is higher than the level of USF2, and they may interact with the *APC* promoter as a heterodimer. However, overexpression of either USF1 or USF2 activates the *APC* promoter in the transient expression assay indicating that these factors may function as homo or heterodimers, and may be able to substitute for one another during *APC* gene expression. Consistent with this concept, experiments using USF1 and USF2 knockout mice suggest that USF1 and USF2 play overlapping roles in the regulation of specific target genes [Sirito et al., 1998].

An increased level of *c-myc* mRNA and protein is well documented in colorectal cancer [Erisman et al., 1985; Guillem et al., 1990]. Furthermore, the loss of *APC* gene function is correlated with deregulation of the *c-myc* gene expression in colorectal and lung cancers [Erisman et al., 1989; Jaiswal et al., 1999]. Recently, a detailed study has been reported examining the role of APC in regulating the *c-myc* gene expression through a β-catenin/Tcf-4 complex [He et al., 1998]. c-Myc, a highly studied oncoprotein, is implicated in the control of cellular proliferation, differentiation, and apoptosis (for reviews, see [Marcu et al., 1992; Zornig and Evan, 1996]). USF1 and USF2 are known to antagonize the effects of c-Myc on cellular proliferation [Luo and Sawadogo, 1996]. Thus, these studies suggest a link between USF1/USF2, APC, and c-Myc. It is tempting to hypothesize that under normal conditions USF1 and USF2 regulate *APC* gene expression, and that an increased APC protein level downregulates β-catenin expression, and thus ultimately *c-myc* gene expression, and that this multi-step process is required for the normal homeostasis of the cell cycle. If *APC* expression is reduced due to impaired function of USF1 and USF2, then the APC protein level is reduced and the entire predicted sequence of events is reversed, resulting in increased *c-myc* expression and its consequences for cell cycle regulation. Future studies to test this hypothesis will enhance our understanding of the role of APC and c-Myc in the progression of colorectal cancer. It will also be of interest to explore the possibility of manipulating *APC* gene expression *via* the USF1 and USF2 transcription factors. These findings may have significant implications for the discovery of potential therapies for colorectal cancer, especially for those cancers involving dysregulation of *APC*.

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REFERENCES

Ahmed Y, Hayashi S, Levine A, Wieschaus E. 1998. Regulation of armadillo by a Drosophila APC inhibits neuronal apoptosis during retinal development. Cell 93:1171–1182.

Aksan I, Goding CR. 1998. Targeting the microphthalmia basic helix-loop-helix-leucine zipper transcription factor to a subset of E-box elements in vitro and in vivo. Mol Cell Biol 18:6930–6938.

Atchley WR, Fitch WM. 1997. A natural classification of the basic helix-loop-helix class of transcription factors. Proc Natl Acad Sci USA 94:5172–5176.

Ayer DE, Kretzner L, Eisenman RN. 1993. Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. Cell 72:211–222

Baeg GH, Matsumine A, Kuroda T, Bhattacharjee RN, Miyashiro I, Toyoshima K, Akiyama T. 1995. The tumour suppressor gene product APC blocks cell cycle progression from G0/G1 to S phase. EMBO J 14:5618–5625.

Behrens J, Jerchow BA, Wurtele M, Grimm J, Asbrand C, Wirtz R, Kuhl M, Wedlich D, Birchmeier W. 1998. Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. Science 280:596–599.

Bello-Fernandez C, Packham G, Cleveland JL. 1993. The ornithine decarboxylase gene is a transcriptional target of c-Myc. Proc Natl Acad Sci USA 90:7804–7808.

Bendall AJ, Molloy PL. 1994. Base preferences for DNA binding by the bHLH-Zip protein USF: effects of MgCl2 on specificity and comparison with binding of Myc family members. Nucleic Acids Res 22:2801–2810.

Blackwell TK, Kretzner L, Blackwood EM, Eisenman RN, Weintraub H. 1990. Sequence-specific DNA binding by the c-Myc protein. Science 250:1149–1152.

Blackwell TK, Weintraub H. 1990. Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science 250:1104–1110.

Blackwood EM, Eisenman RN. 1991. Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science 251:1211–1217.

Boyd KE, Wells J, Gutman J, Bartley SM, Farnham PJ. 1998. c-Myc target gene specificity is determined by a post-DNAbinding mechanism. Proc Natl Acad Sci USA 95:13887–13892.

Browne SJ, Williams AC, Hague A, Butt AJ, Paraskeva C. 1994. Loss of APC protein expressed by human colonic epithelial cells and the appearance of a specific low-molecular-weight form is associated with apoptosis in vitro. Int J Cancer 59:56–64.

Chiang CM, Roeder RG. 1995. Cloning of an intrinsic human TFIID subunit that interacts with multiple transcriptional activators. Science 267:531–536.

Dang CV, Dolde C, Gillison ML, Kato GJ. 1992. Discrimination between related DNA sites by a single amino acid residue of Myc-related basic-helix-loop-helix proteins. Proc Natl Acad Sci USA 89:599–602.

Desbarats L, Gaubatz S, Eilers M. 1996. Discrimination between different E-box-binding proteins at an endogenous target gene of c-myc. Genes Dev 10:447–460.

Dobashi Y, Bhattacharjee RN, Toyoshima K, Akiyama T. 1996. Upregulation of the APC gene product during neuronal differentiation of rat pheochromocytoma PC12 cells. Biochem Biophys Res Commun 224:479–483.

Du H, Roy AL, Roeder RG. 1993. Human transcription factor USF stimulates transcription through the initiator elements of the HIV-1 and the Ad-ML promoters. EMBO J 12:501–511.

Eibner M, Mayer K, Ballhausen WG. 1994. The Human APC gene: transcription regulatory elements within the exon 1A upstream region. Med Genet 6:155.

Erisman MD, Rothberg PG, Diehl RE, Morse CC, Spandorfer JM, Astrin SM. 1985. Deregulation of c-myc gene expression in human colon carcinoma is not accompanied by amplification or rearrangement of the gene. Mol Cell Biol 5:969–1976.

Erisman MD, Scott JK, Astrin SM. 1989. Evidence that the familial adenomatous polyposis gene is involved in a subset of colon cancers with a complementable defect in c-myc regulation. Proc Natl Acad Sci USA 86:4264–4268.

Esteller M, Sparks A, Toyota M, Sanchez-Cespedes M, Capella G, Peinado MA, Gonzalez S, Tarafa G, Sidransky D, Meltzer SJ, Baylin SB, Herman JG. 2000. Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. Cancer Res 60:4366–4371.

Galaktionov K, Chen X, Beach D. 1996. Cdc25 cell-cycle phosphatase as a target of c-myc. Nature 382:511–517.

Gaubatz S, Meichle A, Eilers M. 1994. An E-box element localized in the first intron mediates regulation of the prothymosin alpha gene by c-myc. Mol Cell Biol 14:3853–3862.

Genetta T, Ruezinsky D, Kadesch T. 1994. Displacement of an E-box-binding repressor by basic helix-loop-helix proteins: implications for B-cell specificity of the immunoglobulin heavy-chain enhancer. Mol Cell Biol 14:6153–6163.

Guillem JG, Levy MF, Hsieh LL, Johnson MD, LoGerfo P, Forde KA, Weinstein IB. 1990. Increased levels of phorbin, c-myc, and ornithine decarboxylase RNAs in human colon cancer. Mol Carcinog 3:68–74.

Hart MJ, de los Santos R, Albert IN, Rubinfeld B, Polakis P. 1998. Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. Curr Biol 8:573–581.

He F, Narayan S, Wilson SH. 1996. Purification and characterization of a DNA polymerase beta promoter initiator element-binding transcription factor from bovine testis. Biochemistry 35:1775–1782.

He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. 1998. Identification of c-MYC as a target of the APC pathway. Science 281:1509–1512.

Hiltunen MO, Alhonen L, Koistinaho J, Myohanen S, Paakkonen M, Marin S, Kosma VM, Janne J. 1997. Hypermethylation of the APC (adenomatous polyposis coli) gene promoter region in human colorectal carcinoma. Int J Cancer 70:644–648.

Hodge DR, Thompson DM, Panayiotakis A, Seth A. 1995. Transcriptional activation analysis by the chloramphenicol acetyl transferase (CAT) enzyme assay. Methods Mol Biol 37:409–421.

Horii A, Nakatsuru S, Ichii S, Nagase H, Nakamura Y. 1993. Multiple forms of the APC gene transcripts and their tissue-specific expression. Hum Mol Genet 2:283–287.

Ichii S, Horii A, Nakatsuru S, Furuyama J, Utsunomiya J, Nakamura Y. 1992. Inactivation of both APC alleles in an early stage of colon adenomas in a patient with familial adenomatous polyposis (FAP). Hum Mol Genet 1:387–390.

Jaiswal AS, Kennedy CH, Narayan S. 1999. A correlation of APC and c-myc mRNA levels in lung cancer cell lines. Oncol Rep 6:1253–1256.

Jaiswal AS, Narayan S. 1998. Protein synthesis and transcriptional inhibitors control N-methyl-N′-nitro-N-nitrosoguanidine-induced levels of APC mRNA in a p53-dependent manner. Int J Oncol 13:733–740.

Jones KA, Yamamoto KR, Tjian R. 1985. Two distinct transcription factors bind to the HSV thymidine kinase promoter in vitro. Cell 42:559–572.

Landis SH, Murray T, Bolden S, Wingo PA. 1998. Cancer statistics. CA Cancer J Clin 48:6–29.

Li LH, Nerlov C, Prendergast G, MacGregor D, Ziff EB. 1994. c-Myc represses transcription in vivo by a novel mechanism dependent on the initiator element and Myc box II. EMBO J 13:4070–4079.

Luo X, Sawadogo M. 1996. Antiproliferative properties of the USF family of helix-loop-helix transcription factors. Proc Natl Acad Sci USA 93:1308–1313.

Marcu KB, Bossone SA, Patel AJ. 1992. myc function and regulation. Annu Rev Biochem 61:809–860.

Miyashiro I, Senda T, Matsumine A, Baeg GH, Kuroda T, Shimano T, Miura S, Noda T, Kobayashi A, Monden M, Toyoshima K, Akiyama T. 1995. Subcellular localization of the APC protein: immunoelectron microscopic study of the association of the APC protein with catenin. Oncogene 11:89–96.

Miyoshi Y, Nagase H, Ando H, Horii A, Ichii S, Nakatsuru S, Aoki T, Miki Y, Mori T, Nakamura Y. 1992. Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. Hum Mol Genet 1:229–233.

Morin PJ, Vogelstein B, Kinzler KW. 1996. Apoptosis and APC in colorectal tumorigenesis. Proc Natl Acad Sci USA 93:7950–7954.

Nakamura T, Hamada F, Ishidate T, Anai K, Kawahara K, Toyoshima K, Akiyama T. 1998. Axin, an inhibitor of the Wnt signalling pathway, interacts with beta-catenin, GSK-3beta and APC and reduces the beta-catenin level. Genes Cells 3:395–403.

Narayan S, Jaiswal AS. 1997. Activation of adenomatous polyposis coli (APC) gene expression by the DNA-alkylating agent N-methyl-N′-nitro-N-nitrosoguanidine requires p53. J Biol Chem 272:30619–30622.

Narayan S, Widen SG, Beard WA, Wilson SH. 1994. RNA polymerase II transcription. Rate of promoter clearance is enhanced by a purified activating transcription factor/cAMP response element-binding protein. J Biol Chem 269:12755–12763.

Neufeld KL, White RL. 1997. Nuclear and cytoplasmic localizations of the adenomatous polyposis coli protein. Proc Natl Acad Sci USA 94:3034–3039.

Olson EN. 1990. MyoD family: a paradigm for development? Genes Dev 4:1454–1461.

Olson EN. 1993. Regulation of muscle transcription by the MyoD family. The heart of the matter. Circ Res 72:1–6.

Pena A, Wu S, Hickok NJ, Soprano DR, Soprano KJ. 1995. Regulation of human ornithine decarboxylase expression following prolonged quiescence: role for the c-Myc/Max protein complex. J Cell Physiol 162:234–245.

Prendergast GC, Lawe D, Ziff EB. 1991. Association of Myn, the murine homolog of max, with c-Myc stimulates methylation-sensitive DNA binding and ras cotransformation. Cell 65:395–407.

Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, Vogelstein B, Kinzler KW. 1992. APC mutations occur early during colorectal tumorigenesis. Nature 359:235–237.

Reisman D, Rotter V. 1993. The helix-loop-helix containing transcription factor USF binds to and transactivates the promoter of the p53 tumor suppressor gene. Nucleic Acids Res 21:345–350.

Roy AL, Carruthers C, Gutjahr T, Roeder RG. 1993. Direct role for Myc in transcription initiation mediated by interactions with TFII-I. Nature 365:359–361.

Roy AL, Du H, Gregor PD, Novina CD, Martinez E, Roeder RG. 1997. Cloning of an inr- and E-box-binding protein, TFII-I, that interacts physically and functionally with USF1. EMBO J 16:7091–7104.

Roy AL, Meisterernst M, Pognonec P, Roeder RG. 1991. Cooperative interaction of an initiator-binding transcription initiation factor and the helix-loop-helix activator USF. Nature 354:245–248.

Rubinfeld B, Albert I, Porfiri E, Fiol C, Munemitsu S, Polakis P. 1996. Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. Science 272:1023–1026.

Rubinfeld B, Souza B, Albert I, Muller O, Chamberlain SH, Masiarz FR, Munemitsu S, Polakis P. 1993. Association of the APC gene product with beta-catenin. Science 262:1731–1734.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467.

Sawadogo M. 1988. Multiple forms of the human gene-specific transcription factor USF. II. DNA binding properties and transcriptional activity of the purified HeLa USF. J Biol Chem 263:11994–12001.

Schreiber-Agus N, Chin L, Chen K, Torres R, Rao G, Guida P, Skoultchi AI, DePinho RA. 1995. Cell 80:777–786.

Sirito M, Lin Q, Deng JM, Behringer RR, Sawadogo M. 1998. Overlapping roles and asymmetrical cross-regulation of the USF proteins in mice. Proc Natl Acad Sci USA 95:3758–3763.

Sirito M, Lin Q, Maity T, Sawadogo M. 1994. Ubiquitous expression of the 43- and 44-kDa forms of transcription factor USF in mammalian cells. Nucleic Acids Res 22: 427–433.

Smale ST, Baltimore D. 1989. The "initiator" as a transcription control element. Cell 57:103–113.

Smith KJ, Johnson KA, Bryan TM, Hill DE, Markowitz S, Wilson JK, Paraskewa C, Petersen GM, Hamilton SR, Vogelstein B, Kinzler KW. 1993. The APC gene product in normal and tumor cells. Proc Natl Acad Sci USA 90:2846–2850.

Suzuki S, Adachi A, Hiraiwa A, Ohashi M, Ishibashi M, Kiyono T. 1998. Cloning and characterization of human MCM7 promoter. Gene 216:85–91.

Su LK, Vogelstein B, Kinzler KW. 1993. Association of the APC tumor suppressor protein with catenins. Science 262:1734–1737.

Taso J, Shibata D. 1994. Further evidence that one of the earliest alterations in colorectal carcinogenesis involves APC. Am J Pathol 145:531–534.

Tatsuka M, Ibeanu GC, Izumi T, Narayan S, Ramana CV, Kim N, Kang W, Roy G, Mitra S. 1995. Structural organization of the mouse DNA repair gene, N-methylpurine-DNA glycosylase. DNA & Cell Biol 14:37–45.

Thliveris A, Samowitz W, Matsunami N, Groden J, White R. 1994. Demonstration of promoter activity and alternative splicing in the region 5′ to exon 1 of the APC gene. Cancer Res 54:2991–2995.

Tsuchiya T, Tamura G, Sato K, Endoh Y, Sakata K, Jin Z, Motoyama T, Usuba O, Kimura W, Nishizuka S, Wilson KT, James SP, Yin J, Fleisher AS, Zou T, Silverberg SG, Kong D, Meltzer SJ. 2000. Distinct methylation patterns of two APC gene promoters in normal and cancerous gastric epithelia. Oncogene 19:3642–3646.

Widen SG, Kedar P, Wilson SH. 1988. Human beta-polymerase gene. Structure of the 5′-flanking region and active promoter. J Biol Chem 263:16992–16998.

Weintraub H, Genetta T, Kadesch T. 1994. Tissue-specific gene activation by MyoD: determination of specificity by cis-acting repression elements. Genes Dev 8:2203–2211.

Wong MH, Hermiston ML, Syder AJ, Gordon JI. 1996. Forced expression of the tumor suppressor adenomatosis polyposis coli protein induces disordered cell migration in the intestinal epithelium. Proc Natl Acad Sci USA 93: 9588–9593.

Yasumoto K, Yokoyama K, Takahashi K, Tomita Y, Shibahara S. 1997. Functional analysis of microphthalmia-associated transcription factor in pigment cell-specific transcription of the human tyrosinase family genes. J Biol Chem 272:503–509.

Zervos AS, Gyuris J, Brent R. 1993. Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. Cell 72:223–232. (Erratum: *Cell* 1994, 79, 388.)

Zornig M, Evan GI. 1996. Cell cycle: on target with Myc. Curr Biol 6:1553–1556.