

# Identification of a Cell Type-specific Enhancer in the Distal 5'-Region of the Platelet-derived Growth Factor A-chain Gene\*

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Raymond S. Maul‡, Hongxing Zhang, James D. Reid IV, Nancy G. Pedigo, and David M. Kaetzel§

From the Department of Pharmacology, University of Kentucky Medical Center, Lexington, Kentucky 40536

**Transient transfection analysis of DNA subfragments from the distal 5'-flanking region of the human platelet-derived growth factor A-chain gene (–18.3 to –1.8 kilobase pairs (kb)) revealed enhancer and silencer elements that contribute significantly to transcriptional regulation. Two adjacent regions (–8.2 to –7.5 kb and –7.5 to –7.0 kb) enhanced transcription of both A-chain and heterologous thymidine kinase promoters, whereas repression was observed in two other nearby regions (–9.9 to –8.2 kb and –7.0 to –5.9 kb). The –7.5 to –7.0-kb fragment, or J, was the strongest enhancer, and its activity was localized to a 66-base pair element (A-chain cell type-specific enhancer (ACE 66)). ACE66 activity was highly cell type-specific, with greatest activity seen in choriocarcinoma cell lines (4–10-fold enhancement). Progressive 5'- and 3'-deletions of the ACE66 revealed distribution of activity across the element, with nucleotides 1–33 being critical for function. Electrophoretic mobility shift assays revealed cell type-specific patterns of high affinity protein binding to the element. Ethylation interference footprinting of JEG-3 extract localized guanine contacts on nucleotides 1–18 of both strands of the ACE element, whereas more extensive contacts were made with the phosphate backbone (nucleotides 1–32). The ACE66 element is a potent transcriptional regulator in placental cells and represents a valuable model of long distance regulation in a growth factor gene.**

Platelet-derived growth factor (PDGF)<sup>1</sup> is a potent mitogen and chemoattractant for mesenchymal cells that was first purified from human platelet extracts but later shown to be expressed in a variety of normal and transformed cell types (1, 2). PDGF is a family of dimeric glycoproteins that arise from different combinations of two polypeptide chains, termed A and B, that are encoded by separate genes located on different chromosomes. The human A-chain gene contains 7 exons and is located on chromosome 7 (7pter–7q22), spanning approximately

24 kb (3, 4). Transcription of the A-chain gene is activated rapidly by a wide range of growth factors, cytokines and other mitogens (5–7), whereas complex cell type-specific patterns of A-chain transcription appear to underlie the regulation of cell proliferation during embryogenesis (8) and cellular differentiation (9). A-chain null mice exhibit abnormalities in early embryonic development and formation of lung alveolar myofibroblasts (10, 11), whereas expression of the A-chain and the PDGF  $\alpha$ -receptor also appear to be critical for development of the cardiovascular and nervous systems in mice (12–14). PDGF also appears to be critical for placental development, with high expression of A-chain observed in multiple cell types of the placenta including trophoblasts (2, 15), as well as extraembryonic membranes and uterine smooth muscle cells during pregnancy (15, 16). Inappropriate expression of the PDGF genes has also been implicated in many disease states involving abnormal cell proliferation, including atherosclerosis, pulmonary fibrosis, and neoplasia (17–19).

Analysis of the A-chain promoter region has revealed complex interactions between an array of basal, mitogen-inducible, and silencer DNA elements. A highly GC-rich, S1 nuclease-hypersensitive region (–150 to +1) of the proximal promoter has been shown to contribute most of the basal transcriptional activity in a variety of cell lines, including human cervical carcinoma (HeLa) (20) and renal mesangial cells (21), monkey renal epithelial (BS-C-1) (22), and bovine vascular endothelial cells (23). The –71 to –55 region was shown to mediate phorbol 12-myristate 13-acetate-inducible expression via displacement of the constitutive binding factors Sp1 and Sp3 from the site by Egr-1 (23, 24). Other positive regulatory regions thus far identified include a serum response element (–477 to –468) (20) and a nearby enhancer sequence (–496 to –486) (25). The promoter is also under strong repressive influence of at least two silencer elements, one located within the first intron (26) and the other in the 5'-flanking region (5' S1 nuclease-hypersensitive site) (27). Nevertheless, elements mediating cell type-specific enhancement of transcription have yet to be identified within the –1800 to +8 region of the A-chain gene, suggesting that important regulatory elements may reside in more distally located DNA sequences. In this study, subfragments of the 5'-flanking region from –18 to –1.8 kb were analyzed by transient transfection in a panel of cell lines representing a wide range of A-chain expression levels. A cluster of positive and negative regulatory elements has been identified in a region between approximately –10 and –6 kb, including a 66-bp enhancer (ACE66) that exhibits potent, cell type-specific enhancer activity in choriocarcinoma cells. We further show by electrophoretic mobility shift assay and ethylation interference footprinting that most of this activity in choriocarcinoma cells is mediated through binding of nuclear proteins to direct repeats of AP-1-like sequences within the 5'-portion of the ACE66 element. These findings represent the first detailed analysis of the distal 5'-flanking region of the A-chain gene and

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF085245.

‡ Current address: Division of Hematology-Oncology, Factor 10-240, UCLA School of Medicine, 10833 Le Conte Ave., Los Angeles, CA 90024.

§ To whom correspondence should be addressed: Dept. of Pharmacology, University of Kentucky Medical Center, MS305, 800 Rose St., Lexington, KY 40536-0084. Tel.: 606-257-6558; Fax: 606-323-1981; E-mail: dmkaetz@pop.uky.edu.

<sup>1</sup> The abbreviations used are: PDGF, platelet-derived growth factor; ACE, A-chain cell-specific enhancer; bp, base pair(s); cat, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; kb, kilobase pair(s); luc, luciferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

demonstrate its importance to cell type-specific patterns of A-chain transcription.

#### EXPERIMENTAL PROCEDURES

**Plasmid Constructs and DNA Sequencing**—Construction of the plasmids pAC261 and pUTKAT3 has been described (22, 27, 28). pAC261 contains a -261 to +8 fragment of the A-chain gene that mediates basal promoter activity, whereas pUTKAT3 contains the proximal promoter region of the thymidine kinase gene from herpes simplex virus (-150 to +150). To localize DNA elements in the far upstream region of the 5'-flanking region of the human PDGF A-chain gene, the -18.3 to -3.4-kb region from pACCATX-1 and the -5.2 to -1.8-kb region from pACCATX-11 (3) were subdivided into 15 fragments designated E through S by digestion with restriction endonucleases (Fig. 1). Specifically, E is a 1.65-kb *KpnI-KpnI* fragment; F is a 0.8-kb *SstI-SstI* fragment which overlaps with E; G is a 1.5-kb fragment derived from the 5'-end of pACCATX-11 and an *SstI* site; H is a 0.95-kb *BglII-BssHII* fragment which overlaps with G; I is a 1.1-kb *EcoRI-BglII* fragment; J is a 0.5-kb *XbaI-EcoRI* fragment; K is a 0.75-kb *XbaI-XbaI* fragment; L is a 1.7-kb *BglII-XbaI* fragment; M is a 1.8-kb *EcoNI-BglII* fragment; N is a 0.8-kb *EcoNI-EcoNI* fragment; O is a 0.5-kb *BglIII-EcoNI* fragment; P is a 1.1-kb *BglII-BglII* fragment; Q is a 1.65-kb *BglII-BglII* fragment; R is a 0.95-kb *KpnI-BglII* fragment; and S is a 1.7-kb *SalI-KpnI* fragment derived from the 5'-end of pACCATX-1. The 15 fragments were inserted in both orientations (+/-) into the *XbaI* site of pUTKAT3. Fragments L, K, J, and I were inserted in both orientations into the *XbaI* site of pAC261, whereas fragment J was also inserted in both orientations into a *BamHI* site located 3' to the *cat* gene (via an *XbaI* adapter) of a modified pAC261. Deletions of fragment J (-7.4 to -6.9 kb) were generated either by restriction endonuclease digestion of internal sites or by using standard polymerase chain reaction methods to amplify specific sequences. These polymerase chain reaction fragments contained 209 bp (J209) and 66 bp (J66) of the 3'-end of fragment J. The JΔBsp/pAC261 construct was made by using Klenow enzyme to blunt the ends generated by *BspMI* prior to re-ligation. pGL261-*luc* was constructed by inserting an *XhoI-HindIII* fragment of the PDGF A-chain promoter (-261 to +8) into pGL3-Basic (Promega), a promoterless plasmid containing the luciferase reporter gene. Wild type ACE (J66) was inserted in the sense orientation into the *NheI* site of pAC261-*luc*. J<sup>+</sup>/pAC261-*luc* was constructed by inserting the *BamHI-HindIII* cassette containing fragment J and the A-chain promoter from J<sup>+</sup>/pAC261 into pGL3-Basic. Oligodeoxynucleotides were synthesized (Integrated DNA Technologies) for deletion and mutational analysis of the ACE element. Complementary strands were phosphorylated, annealed, and inserted into the *NheI* site of pAC261-*luc*. Sequence integrity of deletion mutants of fragment J and the ACE element was confirmed by double-stranded DNA sequencing via the Sequenase procedure (U. S. Biochemical Corp./Amersham Pharmacia Biotech) or by an automated fluorescent DNA sequencer (Applied Biosystems).

**Cell Culture and Transient Transfection**—BeWo, BS-C-1, CV-1, HepG2, Hep 3B, JAR, JEG-3, Saos-2, and U2-OS cells were obtained from American Type Culture Collection. HeLa S3 cells were kindly provided by Dr. Anuradha Ray (Rockefeller University). All cells except BeWo, JAR, and Saos-2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal bovine serum (FBS), 10 mM nonessential amino acids, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Saos-2 cells were maintained in the above supplemented Dulbecco's modified Eagle's medium containing 10% (v/v) FBS. BeWo cells were maintained in Ham's F12K medium supplemented with 15% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. JAR cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Plasmids used in transient transfection were purified by an ion-exchange chromatographic procedure (Qiagen). BeWo, BS-C-1, CV-1, HepG2, Hep 3B, JAR, and JEG-3 cells were transfected by calcium phosphate/DNA co-precipitation as described previously (22, 29). HeLa S3, Saos-2, and U2-OS cells were transfected with Dospier liposomal reagent (Boehringer Mannheim). Cells were co-transfected with either RSV-β-gal (22) or CMV-β-gal (30) to correct for variation in transfection efficiency. Assays of *cat* and β-galactosidase activity were performed on cell lysates as described (22, 31, 32). All transfections were performed with duplicate dishes in at least three independent transfection experiments.

**Northern Blot Analysis**—Total cellular RNA was isolated from confluent cultures of cells as described (33). Poly(A)<sup>+</sup> RNA was prepared using oligo(dT)-conjugated magnetic beads (PolyAtract, Promega).

Methods for RNA electrophoresis and blot transfer have been described elsewhere (34). <sup>32</sup>P-labeled DNA probes specific for PDGF A-chain and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were prepared with a nick translation kit (Promega). Probe hybridization, washing of blots, and autoradiography were conducted as described (35).

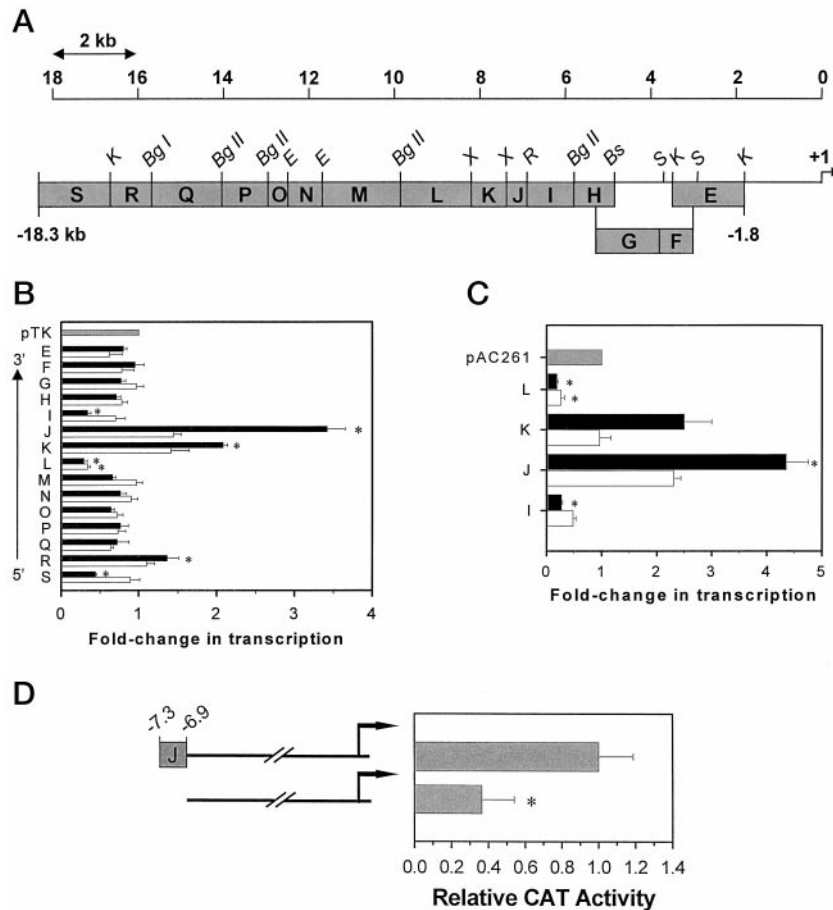
**Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay**—Nuclear extracts were prepared by the method of Dignam *et al.* (36). Electrophoretic mobility shift assays (EMSA) were conducted as described using 10 μg of nuclear extract protein (27). Protein-bound and free DNA were separated by non-denaturing polyacrylamide gel electrophoresis using a 0.5× TBE buffer system. Oligonucleotides corresponding to consensus binding sites reported for AP-1 and ETS-1/PEA3 (37, 38) were employed as unlabeled DNA competitors. A 34-bp oligodeoxynucleotide containing eight consecutive ACGT repeats was utilized as an irrelevant competitor.

**Ethylation Interference Footprinting**—<sup>32</sup>P-End-labeled DNA probes were modified with *N*-ethyl-*N*-nitrosourea as described previously (39). Binding reaction mixtures were scaled up from EMSA conditions by approximately 80-fold (800 μg of JEG-3 nuclear extract protein, 800 μg of poly(dI-dC), 0.3 pmol of probe). Non-denaturing electrophoresis was conducted as described above for EMSA, followed by autoradiography and electroelution of protein-bound and free DNA. DNA was precipitated in the presence of 5 μg of carrier tRNA by addition of sodium acetate, pH 5.2, and ethanol to final concentrations of 300 mM and 70%, respectively. Pellets were rinsed with 70% ethanol, dried, and hydrolyzed at 90 °C for 30 min with either 143 mM NaOH or 1 M piperidine (90 °C, 30 min). NaOH cleavage products were prepared for analysis on sequencing gels by precipitation with sodium acetate and ethanol, a rinse with 70% ethanol, and drying under vacuum. Piperidine cleavage products were dried under vacuum and subjected to three cycles of resuspension in H<sub>2</sub>O and drying. Cleaved fragments were resuspended in formamide loading buffer and resolved by electrophoresis through 12% polyacrylamide sequencing gels, followed by autoradiography at -80 °C with intensifying screens for 24 h.

#### RESULTS

**Identification of Positive and Negative Regions in the Far Upstream Region of the PDGF A-chain Gene**—To identify positive and negative regulatory sequences in the region from -18.3 to -1.8 kb of the PDGF A-chain gene, the region was subdivided into 15 fragments designated fragments E through S (Fig. 1A) and inserted upstream of the thymidine kinase promoter (*tk*) and *cat* reporter gene. Transient transfection analyses were initially performed in HepG2 cells, which express significant amounts of PDGF-A transcripts under basal cell culture conditions (3, 40). Four adjacent fragments L, K, J, and I, which span the region between approximately -10 and -6 kb, conferred significant modulatory effects on *tk* promoter activity (Fig. 1B). Fragment J exhibited the highest enhancer activity of all fragments tested, increasing *cat* activity by 3.4-fold when inserted in the correct orientation and 1.5-fold in the opposite orientation. A lesser degree of enhancement was also observed with fragment K, whereas fragments I and L significantly inhibited transcription. The four fragments had very similar activities when linked to a minimal A-chain gene promoter fragment (-261 to +8) in the plasmid pAC261 (27) (Fig. 1C). When analyzed in JEG-3 (human choriocarcinoma) cells, deletion of fragment J from the 5' terminus of the full-length -7.5 to +8 promoter region caused a 64% decrease in transcription (approximately 3-fold effect), consistent with long distance enhancer activity (Fig. 1D). Fragment J also enhanced transcription 2-4-fold in JEG-3 cells when placed in either orientation downstream of the *cat* gene, a distance of approximately 2 kb from the transcription start site (data not shown). The nucleotide sequence of fragment J was determined and is shown in Fig. 2. Taken together, these results indicate that the distal 5'-flanking region of the A-chain promoter contains multiple enhancer and silencer elements and that the potent enhancer fragment J can act at a long distance from the transcription start site.

**Fragment J Is a Cell Type-specific Enhancer**—To determine whether the fragment J enhancer functions in a cell type-



**FIG. 1. Identification of transcriptional elements in the far upstream region of the PDGF A-chain promoter.** A, summary of relative sizes and positions of fragments (designated E–S, in shaded areas) from the far upstream region of the PDGF A-chain gene that were analyzed for basal transcriptional activity, with the transcriptional start site (+1) indicated with an arrow. Shown at top of panel is a reference scale in kb. Also shown are relevant restriction endonuclease cleavage sites, abbreviated as follows: K, *KpnI*; BgI, *BglI*; BgII, *BglII*; E, *EcoNI*; X, *XbaI*; R, *EcoRI*; Bs, *BssHII*; S, *SstI*. B, shown are the results of transient transfection of HepG2 cells with plasmids containing fragments E–S, inserted 5' of the heterologous HSV-*tk* promoter (pTK); or C, fragments I–L inserted 5' of the minimal A-chain promoter (pAC261). Fold change in transcription represents CAT activities obtained with the indicated promoter fragments (corrected for differences in transfection efficiency by co-transfected  $\beta$ -galactosidase), relative to the activities of pTK or pAC261 alone (gray bars), which are each set to a value of 1. Results obtained with fragments in their correct orientations with respect to the promoter are indicated with black bars, and opposite orientations are shown as open bars. D, A-chain promoter fragments depicted at left were analyzed by transient transfection in JEG-3 choriocarcinoma cells, with relative CAT activity representing observed CAT activity corrected for  $\beta$ -galactosidase activity and normalized to a value of 1 for the -7.5 kb to +8 promoter fragment. Results shown in B–D represent the mean ( $\pm$ S.E.) of three independent transfections. The means are significantly different from each other ( $p < 0.05$ ), as determined by the Student's *t* test.

specific manner, its activity was assessed in a panel of transformed and non-transformed cell lines representing a range of A-chain transcription levels. Fragment J enhanced A-chain transcription by 7.5-fold in JEG-3 cells and 3.8-fold in HepG2 cells when plasmids containing the homologous minimal A-chain promoter and *cat* reporter gene were used (Fig. 3A). A small 2-fold enhancement was seen in the non-transformed monkey renal epithelial line CV-1, whereas activity was undetectable in another cell line of the same type (BS-C-1). When linked to the same minimal A-chain promoter fragment and the luciferase reporter gene, fragment J enhanced transcription by 10-fold in JEG-3 cells (Fig. 3B), similar to the activity observed with the *cat* constructs, and was also highly active in two other choriocarcinoma cell lines, JAR and BeWo. Little or no enhancer activity was seen, however, in Hep3B (hepatocellular carcinoma) or the osteosarcoma lines U2-OS and Saos-2. The potent enhancer activity of fragment J in all three different choriocarcinoma cell lines tested, each of which express high steady-state concentrations of A-chain mRNA (Fig. 3C), strongly suggests a role of the enhancer in maintaining a high rate of A-chain gene transcription in this cell type. The lack of enhancer activity in Saos-2, CV-1, BS-C-1, and HeLa cells is

consistent with previous reports of low A-chain expression (17, 20), as well as our own observations (Fig. 3C).

**Localization of the Fragment J Enhancer to a 66-bp Element**—To localize further the enhancer activity of fragment J, variants were constructed containing deletions at the 5'- and 3'-ends and relocated into pAC261 (Fig. 4A). Although a 100-bp deletion of the 5'-end (J $\Delta$ 5') had no effect on enhancer activity in HepG2 cells, a 400-bp deletion of the 3'-end (J $\Delta$ 3') decreased activity by approximately 50%. An internal deletion of 160 bp near the 3'-end (J $\Delta$ BspMI) resulted in a complete loss in enhancer activity, further localizing the minimal DNA element to the 3'-portion of J. Next, two DNA fragments consisting of the 3'-terminal 209 and 66-bp regions of J were inserted upstream of the minimal A-chain promoter and analyzed by transient transfection in JEG-3 cells (Fig. 3B). Both subfragments displayed full, orientation-independent enhancer activity (3–5-fold), thus localizing the minimal element to the 3'-terminal 66 bp of fragment J. A highly synergistic enhancement of A-chain transcription (100-fold) was observed with three copies of the 66-bp element. In a separate set of transfection experiments conducted across a panel of human tumor cell lines, the minimal element retained the same hierarchy of cell type-specific

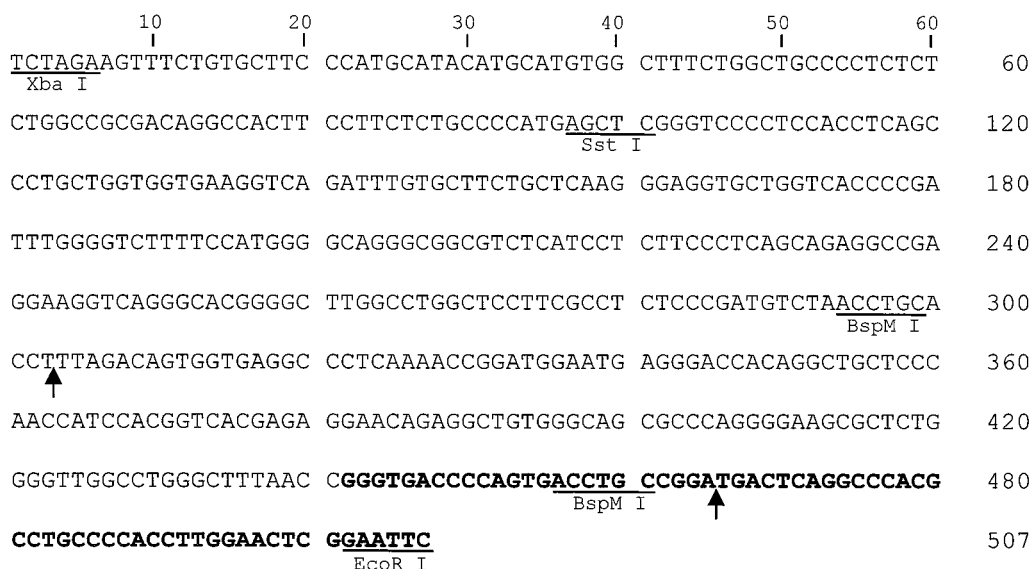


FIG. 2. Nucleotide sequence of fragment J (−7.4 to −6.9) from the human PDGF A-chain gene. The sequence is presented in the 5′- to 3′- direction, with numbers at right indicating the position of the sequence relative to the 5′-terminal nucleotide, designated as 1. Restriction endonuclease sites used to generate deletions are underlined, with cleavage sites for the enzyme BspMI indicated with vertical arrows. Sequence in bold letters (nucleotides 442–507) denotes the ACE66 element. This DNA sequence has been deposited in GenBank™ (accession number AF085245) and has been scanned against the data base; no sequence with significant relatedness was identified.

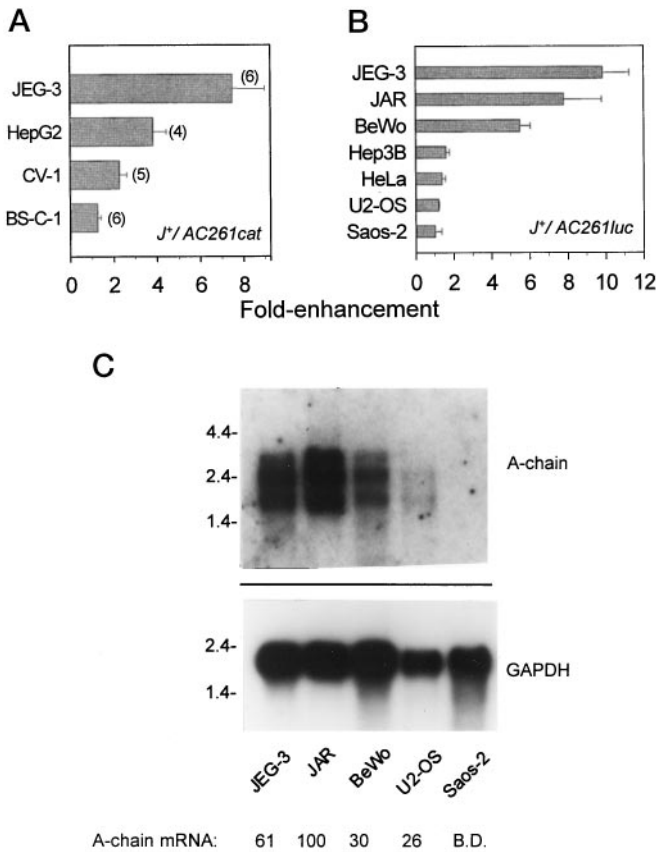
activity seen with the parent fragment J, with highest activities seen in the choriocarcinoma cell lines (4–10-fold), lower levels (~2–3-fold) in HepG2, U2-OS, and HeLa, and no activity in Hep3B and Saos-2 (Fig. 4C). Similar trends of cell type-specific activity were seen with 3 copies of the element (Fig. 4D), with highly synergistic activity seen in the choriocarcinoma cell lines (40–150-fold), less but still synergistic activity in HepG2, U2-OS, and HeLa (15–17-fold), and no activity in Hep3B and Saos-2 cells. Based on the high cell type-dependent activity of the 66-bp enhancer, the element was named A-chain cell type-specific enhancer or ACE66.

**Nucleotide Sequences Distributed across the ACE66 Element Contribute to Its Enhancer Activity**—To identify functionally important subregions of the ACE66 element, a panel of oligodeoxynucleotides introducing 5′- and 3′-end point deletions was designed (Fig. 5A). End points were chosen to correspond with borders of transcription factor binding motifs identified with the MatInspector paradigm (41). When these oligodeoxynucleotides were inserted into pAC261-luc and their activities analyzed by transient transfection in JEG-3 cells, none exhibited the activity of the full-length ACE66 sequence (9.3-fold enhancement; Fig. 5A). Deletion of the ELK-1/E74A consensus sequence from the 3′ terminus of the element (deletion mutant ACE1–54) resulted in a reduction to 5.4-fold enhancement (42% loss). Although deletion of the EF1 consensus element (ACE1–43) had little further effect, removal of the EGR-3 site (ACE1–33) led to an additional loss in activity (2.4-fold enhancement remaining). Deletion of 10 bp from the 5′-end of ACE66 which removed the first AP-1-like site (ACE11–66) resulted in a significant decrease in activity to 3.6-fold. Whereas removal of 12 additional bp from the 5′-end (ACE23–66), which completely deletes the second AP-1-like site and bisects the Ets-1 site, did not diminish enhancer activity further, deletion of the third AP-1-like site (ACE34–66) resulted in almost complete inactivation. Taken together, this analysis demonstrated that DNA sequences distributed over the entire ACE66 element are required for full enhancer activity. Regions contributing to activity were localized to the first and third AP-1-like motifs (regions I and II), the central region containing the EGR-3 motif (region III), and the ELK-1/E74A motif at the 3′-end of the element (IV) (Fig. 5C).

Composite binding sites for ETS and AP-1 (FOS/JUN) transcription factors such as those present in the ACE element have been identified and characterized in other genes and appear to mediate signaling via the Ras-mitogen-activated protein (MAP) kinase pathway (42–44). To assess the contribution made by the potential composite element constituted by the ETS site and the third AP-1 site, point mutations were introduced into the ACE1–43 sequence that disrupted both the “GGA” core of the ETS site (GG → TT) and the AP-1 site (TGACTCA → AGATCTA). When tested in JEG-3 cells, the mutation resulted in a near complete loss of the activity seen with the ACE1–43 sequence (Fig. 5B), indicating the necessity of nucleotides located within the composite element.

**Complex, Cell Type-specific Patterns of Nuclear Protein Binding to the ACE Element, Electrophoretic Mobility Shift Assay**—To identify nuclear factors that bind to the ACE element and mediate its activity, a series of electrophoretic mobility shift assays (EMSAs) were performed with JEG-3 nuclear extract and a radiolabeled oligonucleotide corresponding to nucleotides 1–61 of the ACE element. Incubation of nuclear extract with probe yielded multiple DNA-protein complexes (Fig. 6A, lane 1) that were classified into three groups based on relative electrophoretic mobility (mobility regions 1–3). Bands of mobility region 3 (MR3) were the most prominent, consisting of at least four distinct DNA-protein complexes. A pair of bands of significantly lower intensity was found within MR2, whereas another minor band was seen in MR1. Identical banding patterns were obtained in subsequent EMSAs conducted with a full-length ACE66 probe (data not shown). Addition of unlabeled homologous competitor DNA (ACE1–61) resulted in a dose-dependent displacement of bands in all three mobility regions (lanes 2–4). DNA-protein complexes of MR3 represented high affinity interactions, as indicated by nearly complete displacement with low concentrations of competitor DNA (10-fold molar excess), whereas somewhat higher competitor concentrations (>30-fold) were required to displace complexes in MR1 and MR2. All of the complexes were DNA sequence-specific, indicated by the lack of competing activity of an irrelevant competitor DNA (lane 11).

To localize nucleotide sequences serving as binding sites for the proteins of MR1–MR3, oligodeoxynucleotides containing 5′-



**FIG. 3. Fragment J enhances transcription of the minimal A-chain promoter in a cell type-specific manner.** *A*, the indicated cell lines were transiently transfected with  $J^+$ /pAC261cat or pAC261cat. Fold enhancement represents the increase in CAT activity conferred by fragment J ( $J^+$ /AC261cat). Number of independent transfection experiments for each cell line is shown in parentheses. *B*, transient transfections were conducted in the cell lines shown with the same promoter fragments as in *A*, linked to the luciferase reporter gene (plasmids AC261luc and  $J^+$ /AC261luc). Results represent the mean of at least three independent transfections ( $\pm$ S.E.). *C*, steady-state concentrations of PDGF A-chain mRNA in five human tumor cell lines as assessed by Northern blot analysis. Two  $\mu$ g of poly(A)<sup>+</sup> RNA from the indicated cell lines were subjected to agarose gel electrophoresis, capillary blotting, and hybridization with probes specific for either A-chain (*top*) or GAPDH (*bottom*), as described under "Experimental Procedures." The A-chain and GAPDH blots shown were exposed to x-ray film for 19 days and 21 h, respectively. Estimates of relative A-chain mRNA concentrations were obtained by PhosphorImaging and represent ratios of A-chain to GAPDH mRNA. Values are expressed relative to the A-chain mRNA concentration in JAR cells (arbitrarily set to 100).

and 3'-end point deletions of the ACE66 sequence (see Fig. 5A) were employed as competitors in EMSAs. Each of the 3'-end point-deleted competitors (ACE1-33, ACE1-43, ACE1-54) effectively displaced radiolabeled probe from the MR3 complexes at a 100-fold molar excess (Fig. 6A, lanes 5-7), localizing the DNA-binding site for these proteins to sequence between nucleotides 1 and 33 of the element. This was further demonstrated by the inability of a sequence lacking nucleotides 1-33 to compete (ACE34-66, lane 8). ACE23-66 and ACE11-66, which contain additions of the third and second AP-1 sites, respectively, resulted in slight increases in competing ability compared with ACE34-66 (lanes 9 and 10), but were markedly less potent than the full-length sequence (compare band intensities to those in lane 2). These results indicate that nucleotides 1-11 (AP-1-like site 1) are necessary for assembly of MR3 complexes, with possible additional contributions from nucleotides 12-22 and 23-33. In contrast, the single MR1 complex appears to require binding sites distributed over nucleotides 11-61, as only the ACE1-61 (lanes 3 and 4) and ACE11-66

(lane 10) sequences exhibited significant competing activity. MR2 complexes were most effectively displaced with the sequences ACE1-54, ACE23-66, and ACE11-66, thus localizing the MR2 recognition site to nucleotides 23-54.

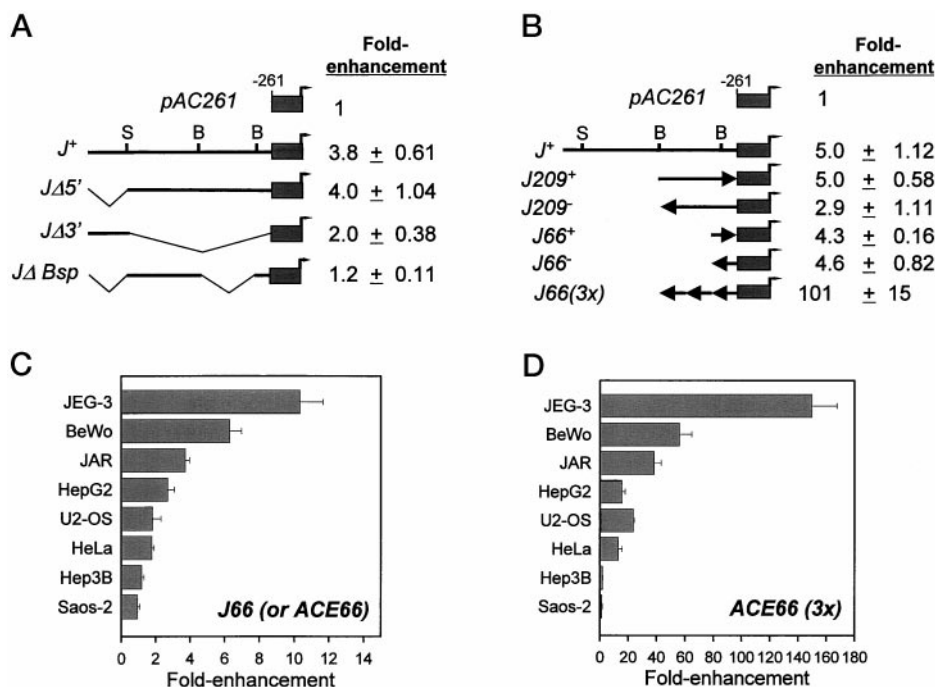
**Enhancer-active and Enhancer-inactive Cells Exhibit Distinct Patterns of Nuclear Protein Binding to the ACE Element**—To assess whether an activated state of the ACE element was associated with a characteristic pattern of protein-DNA complex formation, EMSA analysis was conducted with nuclear extracts obtained from cell lines exhibiting a range of ACE activity. Extracts from cells in which the ACE element was highly active ("enhancer-active"; JEG-3, BeWo, JAR) all produced complexes of primarily the MR3 type. In this study, the minor MR1 band was present only in JEG-3 extracts and could be seen only when long autoradiographic exposure times were employed (data not shown). In contrast, MR2 complexes were produced in marked excess over MR3 in the enhancer-inactive cell lines Saos-2 and BS-C-1. HepG2 and HeLa cells, which exhibit low-to-moderate levels of ACE activity, represented an intermediate state in which MR3 complexes predominated but were present in the context of significant MR2 formation. Taken together, these results demonstrate cell type-specific patterns of protein binding to the ACE element and suggest that net enhancer activity may be determined by competition between stimulatory proteins (MR3) and inhibitory proteins (MR2) for occupancy of the element.

**MR3 Proteins Make Extensive Contacts with Guanine and Phosphate Backbone Residues in the AP-1-like Sequences of the ACE Element, Ethylation Interference Footprinting Analysis**—To localize more precisely the points of DNA contact by proteins in the MR3 complex, a modification of ethylation interference footprinting was used that permits mapping of both protein-guanine and protein-phosphate contacts in a single experiment (39). The analysis utilized an 86-bp oligonucleotide that contained the ACE66 sequence and 10 bp of additional 5'- and 3'-flanking DNA. Interference was clearly seen at guanine residues located within the first and second AP-1-like sites on both the coding and noncoding strands (Fig. 7A). Phosphate backbone contacts were seen at nearly every nucleotide residue over this region, continuing into the adjacent ETS and third AP-1 sites on the non-coding strand (for summary, see Fig. 7B). Localization of protein-DNA contacts in the MR3 complexes to nucleotides 1-33 within the ACE66 element is consistent with the contact sites suggested by the EMSA/oligodeoxynucleotide competitor analysis and taken together with the functional studies indicates a key role for binding of these proteins in mediating ACE activity in JEG-3 cells.

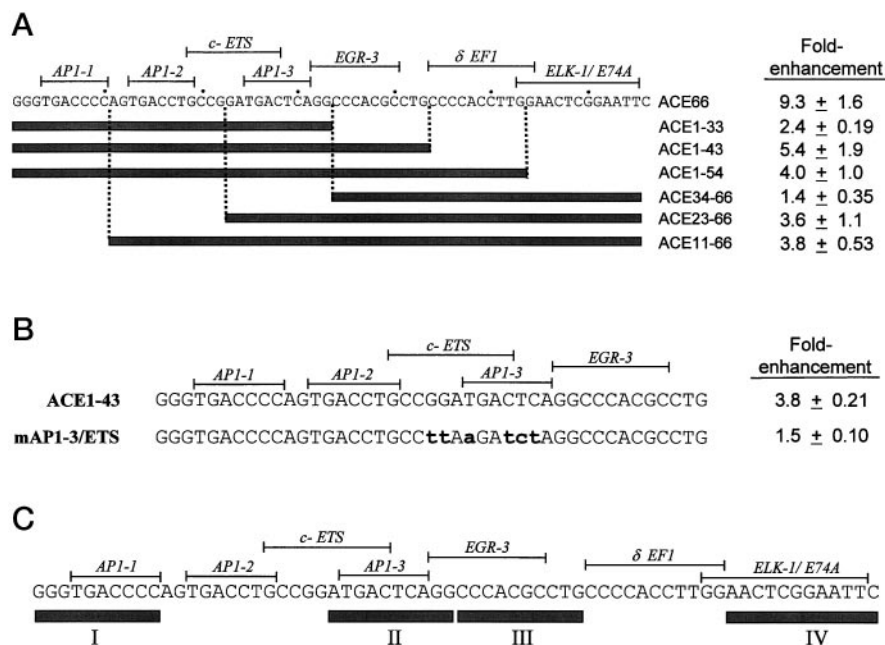
#### DISCUSSION

Activity of the ACE element was highly correlated with high steady-state concentrations of A-chain mRNA across cell lines examined, strongly suggesting an important role for the element in setting transcriptional tone of the A-chain promoter. Of particular interest is its high activity in choriocarcinoma cell lines, which are originally derived from placental tumors of cytotrophoblast origin. PDGF A-chain expression has been shown previously in this cell type *in vivo* (15, 45), where it has been proposed to play an active role in placental growth and development. Although high activity of the ACE in cytotrophoblast-like cells suggests a mechanism for true placenta-specific expression of the gene, the activity could be secondary to the transformed state in choriocarcinoma, where significant potential exists for constitutive activation of signaling pathways that could converge upon the enhancer. Of some relevance is our observation that ACE activity appears to be unaffected in JEG-3 cells by co-transfection with an oncogenic form of Ras<sup>V12</sup> (46) or with Krev-1/Rap1A, an antagonist of Ras function (47)

**FIG. 4. Localization of the cell type-specific enhancer activity of fragment J to a 66-bp sequence by deletional mutagenesis.** *A*, HepG2 cells were transiently transfected with variants of the pJ<sup>+</sup>/AC261cat plasmid containing deletions in fragment J. Fold enhancement is expressed relative to pAC261cat. Shown at *left* are representations of deletions introduced via the indicated restriction endonuclease sites (*S*, *Sst*I; *B*, *Bsp*MI); deleted regions are shown as *thin lines*. *B*, JEG-3 cells were transiently transfected with constructs containing the indicated subfragments of fragment J inserted into pAC261cat in both orientations or as three tandem copies of the 66-bp element (each copy in the opposite orientation). Results shown in *A* and *B* represent the mean of three independent transfections ( $\pm$ S.E.). Shown in *C* is the fold enhancement conferred by the 66-bp subfragment of J (*J66*) in the indicated panel of cell lines following insertion of the element into the pAC261*luc* vector, and *D* summarizes results obtained in the same cell lines with 3 tandem copies of the element (*J66*(3 $\times$ )).



**FIG. 5. End point deletions and internal mutagenesis reveal distribution of enhancer activity across the entire ACE66 element.** *A*, at *top* is shown the nucleotide sequence of the ACE66 element and potential transcription factor binding motifs, based on computer analysis (41). Represented as *shaded boxes* and aligned with the ACE66 sequence are subfragments that were synthesized as oligodeoxynucleotides and inserted in pAC261*luc*. At the *right* of each subfragment is the corresponding fold enhancement observed following transient transfections in JEG-3 cells. *B*, point mutations (*lowercase, bold*) were introduced into ACE subfragment 1-43 at the composite ETS/AP-1 element (mAPI-3/ETS) and their effect on enhancer activity assessed in comparison to the unmodified ACE1-43 sequence. *C*, summary of subregions (I-IV) of ACE66 shown to be required for enhancer activity. Subregions I-IV are indicated by *shaded bars*, immediately below and aligned with the ACE66 sequence.

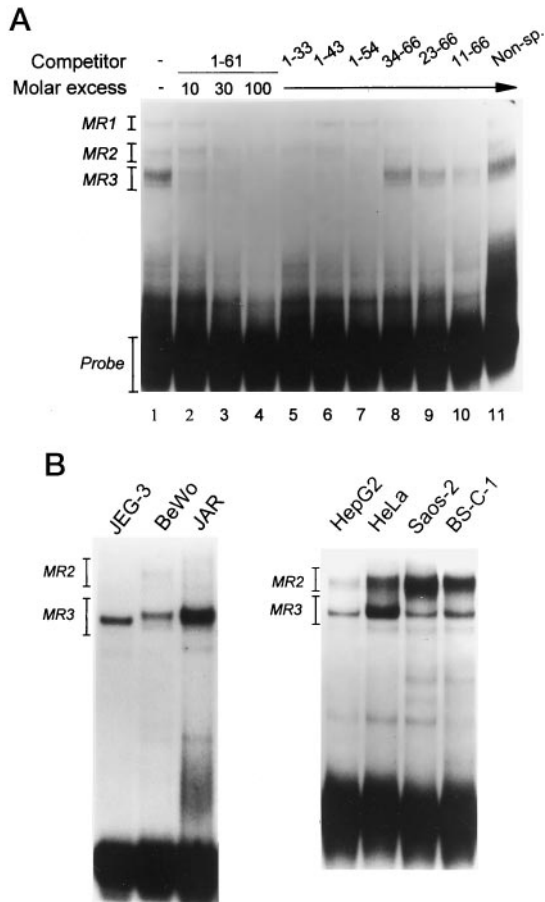


(data not shown). Thus, despite possessing multiple consensus elements for AP-1 and ETS family members, the element does not appear to be a downstream target of the Ras-mitogen-activated protein kinase pathway. In addition to the correlation between ACE activity and A-chain mRNA expression in choriocarcinoma cells, the element does exhibit some activity in other A-chain-expressing cell types such as HepG2 and U2-OS, further suggesting a fundamental role in determination of cell type-specific transcription. This observation is reinforced by the marked cell type-specific activity observed with three tandem copies of the ACE element (Fig. 4D).

Although the direct repeats in the 5'-portion of the ACE66 sequence appear to play a central role in mediating enhancer activity, significant contributions are also made by nucleotides located in the EGR-3 and ELK-1 consensus binding sequences found further downstream. This high degree of complexity within the ACE element suggests potentially complex interac-

tions between cognate ACE-binding transcription factors, as well as increased potential for mediating the multitude of developmental, cell type-specific and inducible signals that stimulate A-chain gene transcription. The Elk-1/E74A sequence contains a perfect match to the ETS core motif (C/A) GGA (A/T) (48) and identity in 10 out of 12 nucleotides of the binding site for E74A, an ETS family member that is induced by ecdysone in *Drosophila* (49). ETS factors often participate in higher order protein-DNA complexes, such as that between Elk-1 and serum response factor, as components of the ternary complex which binds the c-Fos serum response element (50); ETS proteins could be envisioned to interact in similar fashion with factors that bind to the direct repeat and EGR-3 sequences within the ACE element.

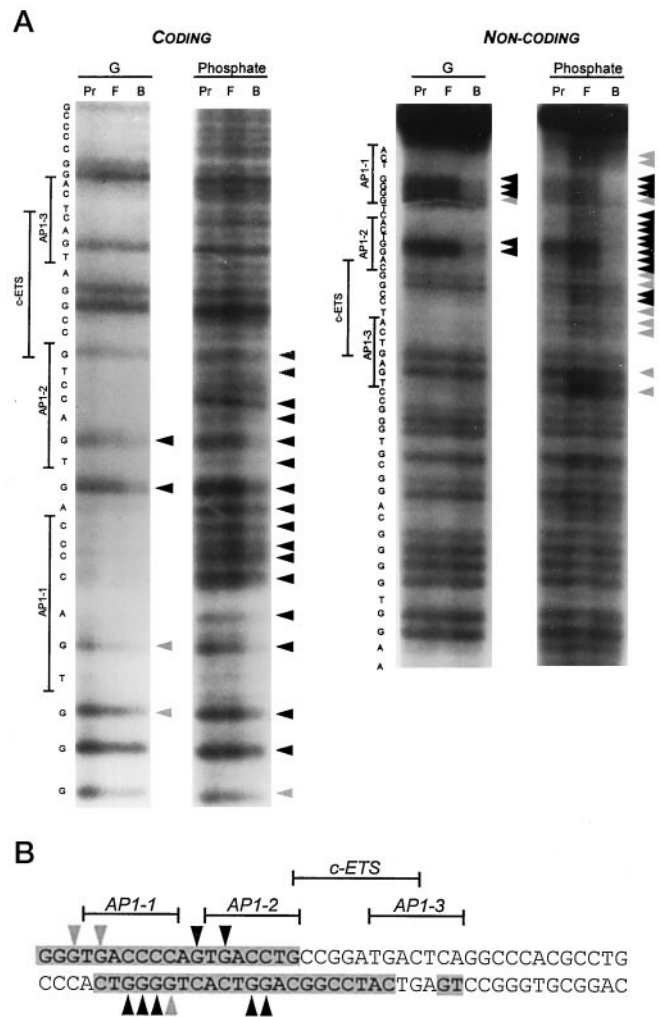
The predominance of EMSA MR2 complexes over MR3 in the enhancer-inactive cells Saos-2 and BS-C-1 suggests a potential role for binding of MR2 proteins in suppression of ACE activity.



**FIG. 6. EMSA reveals cell type-specific patterns of protein binding to the ACE element.** A, EMSA was performed as described under "Experimental Procedures" with nuclear extract (10  $\mu$ g) from JEG-3 cells and radiolabeled ACE probe (ACE1-61). Incubations were conducted in the absence (*lane 1*) or presence of the indicated panel of unlabeled ACE66 oligodeoxynucleotides (*lanes 2-10*), previously described in Fig. 5A. Also included was a 34-bp nonspecific competitor DNA (*Non-sp.*, *lane 11*) consisting of direct repeats of the sequence AGCT. DNA-protein complexes were classified according to their presence in one of three regions of the gel, indicated at *left* with *brackets* as mobility regions 1-3 (*MR1-MR3*). B, EMSA was conducted with nuclear extracts (10  $\mu$ g/lane) from the indicated cell lines and ACE1-61 probe.

In this regard, nucleotides 44-54 of the ACE element are highly homologous to a consensus binding motif for the  $\delta$ -crystallin enhancer-binding protein ( $\delta$ EF-1), which has been identified as a repressor of E2 box-mediated gene activation (51). Repressors such as  $\delta$ EF-1 are proposed to oppose activator proteins, either through competition for overlapping binding sites or by repression of neighboring activation elements (52). Competitive EMSA studies revealed that the binding sites for the MR2 and MR3 complexes do overlap, suggesting the potential for such a regulatory mechanism at the ACE element.

Distal elements have been shown to play critical roles in the regulation of the human globin genes (53), as well as those encoding chicken lysozyme (54),  $\alpha$ -fetoprotein (55), murine syndecan-1 (56), and human PDGF B-chain (57). Interestingly, many of these upstream elements have been implicated in mediating developmental patterns of transcription, suggesting a similar role for the distal regulatory elements of the A-chain gene identified in the current study. Of these, the HS2 enhancer within the human globin gene locus control region represents a very similar and relevant model to the ACE element, as it is located in a far upstream location (-10 kb from the  $\epsilon$ -globin gene), has an obligate requirement for two tandem AP-1 sites, and also possesses a complex arrangement of bind-



**FIG. 7. Ethylation interference footprinting reveals extensive contacts between nuclear proteins from JEG-3 cells and guanine and phosphate backbone residues in the 5'-portion of the ACE66 element.** A, an 86-bp oligodeoxynucleotide containing the ACE66 sequence and 10 additional bp of 5'- and 3'-flanking DNA was asymmetrically end-labeled with  $^{32}$ P on either the coding or non-coding strand and ethylated by incubation with *N*-ethyl-*N*-nitrosourea as described under "Experimental Procedures." Ethylated probes were incubated with JEG-3 nuclear extract, subjected to nondenaturing electrophoresis to separate bound (MR3 bands) and free DNA, followed by cleavage at either guanine (G) residues with piperidine or at phosphate residues with NaOH. Shown are cleavage products obtained with ethylated probe alone (*Pr*), free (*F*), and bound (*B*) probes. Specific points of DNA-protein contact are identified at the *right* of each set of gel lanes using *arrows*, with the relative darkness of the *arrows* indicating degree of interference with binding. B, summary of results shown in A. Guanine residues required for binding of MR3 complex proteins to the ACE66 element are identified with *arrows*; phosphate residues are shown as *bold letters* within *shaded boxes*.

ing sites for other accessory factors such as NF-E2, GATA, YY1, and USF (58, 59). Such an arrangement of multiple protein-binding sites within a single element, particularly those recognized by families of proteins, is typical of elements that control cell type-specific and developmental patterns of gene transcription (reviewed in Ref. 60). The relative proximity of positive and negative regulatory elements far upstream of the A-chain promoter is also reminiscent of similar organization seen in the chicken lysozyme gene, which contains three enhancers (-6.1, -3.6, and -2.7 kb) and one silencer (-2.4 kb), and the PDGF B-chain gene, which also possesses two distal enhancers (-8.6 and -9.9 kb) and one silencer (approximately -11 kb). Clustering of the distal elements of the A-chain gene strongly sug-

gests potential for functional interactions between them, and studies are underway to dissect their hierarchies of importance and potential for cooperativity in different regulatory settings.

We have demonstrated that the distal 5'-flanking sequence of the PDGF A-chain promoter contains multiple positive and negative regulatory elements, one of which (ACE) appears to be important for enhancement of transcription of the A-chain gene in choriocarcinoma cells. Moreover, the correlation observed between ACE activity and A-chain expression across a panel of transformed and non-transformed cell lines suggests a fundamental role for this element in determining cell type-specific patterns of A-chain gene transcription, such as those elicited in response to developmental and mitogenic signals.

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