The Human α2 Integrin Gene Promoter
IDENTIFICATION OF POSITIVE AND NEGATIVE REGULATORY ELEMENTS IMPORTANT FOR CELL-TYPE AND DEVELOPMENTALLY RESTRICTED GENE EXPRESSION*

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Mary M. Zutter†‡, Samuel A. Santoro‡, Audrey S. Painter‡, Ying Li Tsung†, and Alex Gafford†
From the Departments of †Pathology and ‡Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

The α2β1 integrin serves as a collagen receptor or a collagen/laminin receptor, depending upon cell type. Expression of the integrin is regulated during normal cellular differentiation and is altered during carcinogenesis. We have previously demonstrated that increased expression of the α2β1 integrin during megakaryocytic differentiation is a consequence of increased α2 mRNA due to transcriptional activation of the α2 integrin gene and that the decreased expression of the integrin in breast adenocarcinomas is due to decreased steady-state levels of α2 mRNA. We now report the identification and characterization of the 5'-flanking region of the α2 integrin gene. The 5'-untranslated region of the α2 mRNA extends 129 base pairs 5' to the site of translation initiation. The promoter region lacks TATA and CAAT boxes but contains an abbreviated initiator sequence and six Sp1 binding sites. Consensus binding sites for AP-1 and AP-2 complexes, a GATA box, a Pu.1 box, and two palindromic motifs with potential to bind the estrogen receptor are also present. A 961-base pair fragment of the 5'-flanking region directs both cell-type and differentiation-specific expression of a reporter gene in T47-D epithelial cells and in pluripotent hematopoietic K562 cells upon megakaryocytic differentiation.

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§To whom correspondence should be addressed: Dept. of Pathology, Box 8118, Washington University School of Medicine, 660 South Euclid, St. Louis, MO 63110. Tel.: 314-362-0114; Fax: 314-361-4909 or 362-8950.

The integrin superfamily of heterodimeric adhesive protein receptors mediates adhesive interactions between cells and between cells and the extracellular matrix (1–3). The regulation of integrin gene expression is thought to be important in complex biological events of cell differentiation, migration, wound healing, and altered adhesive and invasive properties of tumor cells (4–7). The α2β1 integrin, also known as the platelet membrane Ia-IIa complex, the very late activation antigen-2, and the Class II extracellular matrix receptor serves as a collagen receptor on platelets and fibroblasts but as a collagen and laminin receptor on many epithelial cells and on endothelial cells (8–13). Our immunohistochemical studies revealed both cell-type and differentiation-specific expression of the α2β1 integrin (14). The α2 subunit is expressed on many epithelial cell types including those of the skin, breast, and colon, as well as on fibroblasts, endothelial cells, megakaryocytes, platelets, and activated T cells. The contrast of high-level expression of the α2β1 integrin by the proliferative layers of squamous epithelium with the absence of α2 expression by the more differentiated superficial epithelial cell layers is one striking example of differentiation-dependent regulation of the α2β1 integrin (14). The normal high-level expression of the α2β1 integrin by the luminal epithelium of ducts and ductules of normal human breast tissue is reduced in moderately differentiated adenocarcinomas of the breast and is more markedly diminished in poorly differentiated carcinomas (15).

To begin to discern the molecular mechanisms by which both the cell type and differentiation-dependent expression of the α2β1 integrin are regulated, we have employed a model of hematopoietic differentiation. The K562 cell, a leukemic cell line derived from a patient with chronic myelogenous leukemia in blast crisis, represents a pluripotent hematopoietic cell (16). K562 cells cultured in the presence of 40 nM phorbol dibutyrate acquire megakaryocytic characteristics, including expression of the α2β1 and α2β3 integrins (17, 18). We have recently demonstrated that the increased surface expression of the α2β1 integrin is a consequence of increased steady-state levels of α2 mRNA due to transcriptional activation of the α2 gene. The long-lived β1 mRNA was not altered during differentiation (19).

We now report the identification of the 5'-flanking region of the α2 integrin gene, compare its structure to the other known integrin promoters, and demonstrate that cell-type-specific promoter activity resides within the 5'-flanking region of the gene.

MATERIALS AND METHODS
Cell Cultures and Transfection Assays—T47-D and K562 cell lines obtained from the ATCC were propagated in RPMI 1640 medium either with or without 0.2 units/ml insulin, respectively. Megakaryocytic differentiation of K562 cells was induced by the addition of 40 nM phorbol dibutyrate in dimethyl sulfoxide, as previously described (18). The K562 cell line was transfected by electroporation using a BTX Electro Cell Manipulator 600 (BTX Inc., San Diego, CA) (20). Approximately 1.0 × 10⁶ cells were transfected in RPMI medium containing 100 µM salmon sperm DNA, 30 µg of plasmid DNA, and 3 µg of RSV-luciferase DNA by electroporation at 275 V and 600 µF. Transfected cells were harvested after 48 h. Luciferase activity produced by 10 µl of the luciferase in 190 µl of assay buffer (10 mM MgOAc₂, 50 mM Tris–MES, pH 7.9, and 2 mM ATP) was analyzed using a Monolight 2010 luciferometer (Analytical Luminescence Laboratory, San Diego, CA), as described (21), and was used to normalize for transfection efficiency. Cell extracts containing identical amounts of luciferase activity were then assayed for chloramphenicol acetyltransferase (CAT) activity by the standard method of Goren et al. (22). The conversion of chloramphenicol to acetylated chloramphenicol was determined by both thin-layer chromatography and differential extraction.

1 The abbreviations used are: RSV, Rous sarcoma virus; MES, 4-morpholinonephane sulfonic acid; PMSF, 1,4-piperazinediethanesulfonic acid; bp, base pairs; kb, kilobase pairs; CAT, chloramphenicol acetyltransferase; Inr, initiator sequence.

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FIG. 1. Partial restriction enzyme map of exon-1 and the 5'-flanking region of the α2 integrin gene. The top line demonstrates the approximate length of the Sau3A fragment from the two identical bacterial phage clones (AG0-1 and AG0-2). The internal 2.0-kb EcoRI genomic fragment hybridized with the 115-bp 5'-partial cDNA probe and the oligonucleotide probe (α2012). Comparative sequence analysis of the 2.0-kb EcoRI genomic fragment identified the 5'-untranslated region and exon 1, to the first intron. The arrow at +1 denotes the most 5' cluster of transcription start sites. The identified AvaII site which occurs 109 bp downstream in the 5'-untranslated region was utilized for construction of the S1 nucleic acid protection probe as well as the CAT α2 promoter-reporter gene constructs.

cDNA and Genomic Cloning—Phage clones containing the complete α2 cDNA were obtained from a human placental AGTII cDNA library, graciously provided by Dr. Evan Sadler (29). Genomic clones were isolated from a human lung fibroblast genomic library consisting of MboI partially digested DNA cloned into AFix (Stratagene, La Jolla, CA). Both genomic and cDNA libraries were screened with both a 5'-labeled 115-bp EcoRUDraI α2 cDNA fragment (extending from position -47 to +71) and a 5'-labeled oligonucleotide (extending from position -5 to +16) of the published α2 cDNA sequence (24). These positions are given in relation to the ATG methionine of the published α2 cDNA sequence. Two identical positive clones (AG0-1 and AG0-2) were obtained. Partial restriction enzyme mapping of positive phase clones was performed by standard techniques with single and double restriction enzyme digestion and Southern blot analysis (25). Blasts were hybridized overnight to radiolabeled cDNA probes prepared by the random-primer method or oligonucleotide probes prepared by end labeling with T4 polynucleotide kinase and 32P by standard techniques with single and double restriction enzyme digestion and Southern blot analysis (25). Probe binding, and two partially-conserved CAAT-binding transcription factors are boxed and labeled. These include binding sites for ubiquituous cell type-specific, and differentiation-dependent transcription factors, including Sp1, AP-1, and AP-2 sites, a GATA and a Pu site.

FIG. 2. The nucleotide sequence of the 5'-end, 5'-flanking region, and exon-1 of the α2 integrin gene. Exon 1 extends from the most 5' cluster of transcription start sites, denoted by +1, to the intron-exon border, denoted by an arrow. The amino acid sequence is shown below the coding region of exon 1. Consensus sequences for binding to transcription factors are boxed and labeled. These include binding sites for ubiquitous cell type-specific, and differentiation-dependent transcription factors, including Sp1, AP-1, and AP-2 sites, a CAT and a Pu box, two half-palindromic motifs with potential for estrogen receptor binding, and two partially-conserved CAAT-binding transcription factor-NF-1 sites. The conserved TCA sequence of the Inr at the transcription start site is underlined with a solid line. The position of the oligonucleotide (α2012) used for primer extension analysis and sequencing is underlined with a dashed line.

poly(A)* RNA for 16 h at 35 °C in the hybridization buffer described above. The complementary DNA strand was extended with murine mammary tumor virus reverse transcriptase at 42 °C for 2 h. The length of the extended products was determined by sequencing the α2 genomic fragment using the same primers.

α2 CAT Fusion Constructs—The promoter and enhancer activity of the 5'-flanking region was analyzed by inserting the EcoRI-AvaII genomic fragment (-961 to +109) in relation to the transcription start site at +1) upstream to CAT structural sequences in the CAT expression vector pCAT-Basic (Promega, Madison, WI), which lacks natural CAT regulatory elements. Nucleotide deletion mutants of the 1.0-kb construct (pα961-CAT) were generated by restriction enzyme digestion of pα961-CAT at the restriction enzyme sites PstI (-30), XmnI (-92), BglII (-549), and AccI (-776). Cytochalasin-D-CAT containing the cytochalasin-D promoter directing transcription of the CAT gene served as a positive control. All transfection efficiency in all assays. All transfection experiments were performed at least four times.
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RESULTS AND DISCUSSION

An α₂ cDNA Clone Extends the 5' Untranslated Region—The 8-kb α₂ mRNA is approximately 3.0 kb larger than the published sequence of the α₂ cDNA (24). To determine the length and sequence of the 5'-untranslated region, three additional α₂ cDNA clones were identified by screening a human placenta Agt11 cDNA library with a 118-bp α₂ cDNA, which represented the 5'-end of the published sequence (23, 24). The longest of the three clones was partially characterized by restriction enzyme digestion, Southern blot hybridization, and sequence analysis. The 5'-untranslated region of the longest clone extended 129 bp 5' to the initiation methionine; this site was initially designated +1 (Fig. 1), and data is presented below to verify that this site represents one of several heterogeneous 5'-ends of the α₂ cDNA. We therefore concluded that the 3'-untranslated region of the α₂ mRNA extends approximately 3 kb beyond the end of the published cDNA sequence.

Isolation of a 5' α₂ Genomic Clone—Current models of gene regulation propose that specific DNA protein interactions regulate developmental and tissue-specific gene transcription. The promoters and enhancers of many genes including those of the integrin receptors α₁β₂, α₄β₁, CD11a, and CD11c, are located in 1.0 to 2.0 kb of the 5'-flanking region of the respective gene (29–36). To further characterize the structure of the 5'-untranslated and 5'-flanking regions of the α₂ gene, 5 x 10⁶ independent bacteriophage clones from a bacteriophage library derived from human lung fibroblast genomic library in λFix (Stratagene, La Jolla, CA) were screened with the 118-bp α₂ cDNA clone and an oligonucleotide probe (α₂012). Two bacteriophage clones (AGα₂-1 + λGα₂-2) were shown to be identical by partial restriction enzyme mapping and Southern blot hybridization (Fig. 1). Both λGα₂-1 and λGα₂-2 contained a 2.0-kb EcoRI restriction fragment which hybridized to both partial cDNA and oligonucleotide probes. Comparative sequence analysis of the 2.0-kb EcoRI genomic fragment and the α₂ cDNA clone described above revealed 192 bp of identity beginning at +1 of the cDNA and extending to the border of the first intron; the relationship of the genomic clones to the cDNA is shown in Figs. 1 and 2. The 2.0-kb EcoRI genomic fragment contains 961 bp of 5'-flanking sequence, all of exon 1 with 129 bp of 5'-untranslated region and 64 bp of coding sequence, and part of the first intron (Fig. 2). The genomic sequence diverged from the cDNA at the intron-exon border of the first intron. The sequence flanking the +1 transcription start site lacked consensuses sequences for splice donor or splice acceptor sites.

Mapping the Site of Transcription Initiation—To map the site of transcription initiation, primer extension and S1 nuclease analysis were used in combination. S1 nuclease analysis, performed using a 5'-end-labeled EcoRI-AvaII genomic fragment (extending from −961 to +109) and hybridized to total mRNA from K562 cells induced with phorbol dibutyrate for 8 days, revealed two sets of protected fragments, 92–99 and 109–119 nucleotides in length (Fig. 3). Protected fragments were not
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**A**

Diagram of α2 integrin promoter-CAT constructs. The construct pα2961-CAT contained the entire 5'-flanking region shown in Figs. 1 and 2, with 109 bp of 5'-untranslated region upstream to the CAT structural gene. Constructs pα2776-CAT, pα2549-CAT, pα292-CAT, and pα230-CAT were deletion mutants derived from pα2961-CAT. B, α2 integrin promoter activity in the epithelial cell line, T47-D. The constructs pα2961-CAT, pα2776-CAT, pα2549-CAT, pα292-CAT, and pα230-CAT were transfected in parallel with cytomegalovirus-CAT, which contains the strong viral promoter, and

**B**

Bar graph showing relative CAT activity for different constructs in T47-D cells.

**C**

Western blot analysis of K562 cells in an uninduced state and upon induced differentiation (megakaryocytic).

FIG. 5. A, diagram of α2 integrin promoter-CAT constructs. The construct pα2961-CAT contained the entire 5'-flanking region shown in Figs. 1 and 2, with 109 bp of 5'-untranslated region upstream to the CAT structural gene. Constructs pα2776-CAT, pα2549-CAT, pα292-CAT, and pα230-CAT were deletion mutants derived from pα2961-CAT. B, α2 integrin promoter activity in the epithelial cell line, T47-D. The constructs pα2961-CAT, pα2776-CAT, pα2549-CAT, pα292-CAT, and pα230-CAT were transfected in parallel with cytomegalovirus-CAT, which contains the strong viral promoter, and
observed with mRNA from uninduced K562 cells which express minimal α2 mRNA or REH cells, a lymphoblastic cell line which does not express α2 mRNA (Fig. 3). These results suggest the existence of multiple transcription initiation sites, with two major sites located 112 and 129 bp upstream of the initiation methionine. The 5'-end of the full-length cDNA described above, designated +1, terminated at the same site as the 109-bp fragment protected in the S1 nuclease analysis. Primer extension analysis using the antisense oligonucleotide α2012 (Fig. 4) identified multiple extension products whose sizes predicted transcription initiation sites identical to those determined by S1 nuclease analysis. These findings confirmed the presence of multiple transcription initiation sites in two clusters between 112 and 129 bp upstream of the initiation methionine.

The 5'-Flanking Region of the α2 Gene—The 5'-flanking sequence around the site of transcription initiation of the α2 gene resembles that of other integrin promoters by lacking both TATA and CAAT boxes, as shown in Fig. 2 (29–36). The site of transcription initiation for the other integrin promoters which lack TATA or CAAT boxes is a consensus sequence defined as the initiator sequence (Inr) (129, 32, 33, 36). The Inr consensus sequences for α2, αβ2, CD11a, and CD11b resemble closely the original consensus sequence of the terminal deoxynucleotidyl transferase gene (37). The sequence 5′-CCCTGCTC=C-GG-3′ at the initiation site of the α2 integrin gene maintains the consensus TCA at -2 to +1 and conserves pyrimidine-rich stretches on either side of the transcription start site but diverges somewhat from the Inr sequences of other integrins in both the 3' or 5' directions. The Inr has been shown to direct correct transcription initiation in association with nearby Sp1 sites (38, 39). Within 100 bp of the transcription start site, four consensus sequences for Sp1 are identified, including one Sp1 site between the two clustered sites of transcription initiation. Additional Sp1 sites are located at -291 and -307.

In addition to the Sp1 sites, numerous consensus binding sites for ubiquitous cell type-specific and differentiation-dependent transcription factors are located in the 961-bp segment of the 5'-flanking region. Consensus binding sites for transcription factors include two AP-1 sites at -765 and -702, an AP-2 site at -132, and six AP-2-CAAT sites at -311, -294, -222, -216, -55, and +3. Whereas both AP-1 and AP-2 mediate enhanced transcription via the phorbol ester/dicacylglycerol-activated protein kinase C pathway, AP-2 also mediates enhanced transcription via a CAMP-dependent protein kinase A pathway. Regulatory regions recognized by AP-2 are found in the SV40 enhancer, human growth hormone, c-myc, and histocompatibility H-2Kb genes (40, 41). In addition to consensus sequences for ubiquitous transcription factors, two elements associated with hematopoietic differentiation are adjacent to one another. A consensus site for Pu.1 located at -666 bp of the 5'-flanking sequence binds members of the hematopoietic-specific ets family of protooncogenes (42, 43). A single GATA site at -679 bp may direct differentiation along the erythroid/megakaryocytic and mast cell lineages (44). The adjacent binding sites for ets and GATA transcription factors suggest the possibility of either cooperative interaction or competitive inhibition. Members of the GATA family of DNA-binding proteins have been shown to function in cooperation with CACC motifs found in globin promoters (44) as well as with other DNA-binding proteins in mediating tissue-specific expression of the rat platelet factor 4 gene (45). However, consensus binding sites for Pu.1 and GATA are adjacent to one another in the CD11b promoter, yet only the Pu.1 site is functionally active (46). Two half-palindromic motifs with the potential for estrogen receptor binding are positioned at -514 and -802 (47). We previously observed a correlation between α2β1 integrin expression and estrogen receptor expression in breast cancer (15), raising the possibility that α2 integrin expression may be hormonally regulated. The identification of two estrogen receptor half-sites supports the role for hormonal regulation of the α2 integrin gene. Recently, the estrogen receptor gene was shown to be transcriptionally active in megakaryocytes and in the megakaryocytic cell line SO1 (48), an observation consistent with a role for estrogen in regulating expression of α2β1 and other megakaryocytic genes. Sites for steroid receptor binding have not been identified in other integrin promoters.

In addition to the emphasis placed on the positive regulatory elements required to activate or enhance gene transcription, recent evidence supports the importance of cell type-specific negative transcription factors called silencers. The two partially conserved CTF-NF1 (CCAT-binding transcription factor-NF1) sites at positions -235 and -181 in a gene which lacks both TATA and CAAT box sequences closely resemble the NF1-like consensus identified in the rat growth hormone gene, the human retinol-binding protein, the mouse Sparc (osteonectin) gene, the chicken β-globin gene, the mouse Ren-1d negative element, and a silencer site in the mouse α2(1) collagen gene (49). In these genes, the NF1-like consensus sequence serves as a negative regulatory element which suppresses gene expression in a cell type-specific manner.

The sequence of the 5'-flanking region between +1 and -400 is composed of 70% guanine (G) and cytosine (C) residues, with 9 HpaII restriction enzyme sites (CCGG) concentrated in the region between +1 and -389. A GC-rich segment with numerous HpaII restriction enzyme sites, also observed in the α2 integrin promoter, suggests that this may be part of an HpaII tiny island fragment, a region which can be regulated by altering chromatin configuration (50). Distal to the GC-rich region, a short segment of dinucleotide repeats extends from -604 to -647. The dinucleotide repeats of TG or CA which compose the majority of the repetitive sequence have been identified in the introns of human γ-globin and cardiac actin genes, and downstream of mouse immunoglobulin genes (51–53). These repetitive elements may induce conformational changes in the DNA helix which result in altered DNA-protein interactions (54, 55).

The α2 Promoters Demonstrate Cell Type-specific Promoter Activity—To determine if the 5'-flanking region of the α2 gene functions to direct transcription in a cell-type and differentiation-dependent manner, the 1070-bp EcoRI-AuII genomic fragment spanning -961 to +109 was placed upstream of a CAT structural sequences in a CAT expression vector (p961-CAT) (Fig. 5A). The promoter activity of the 1070-bp genomic region was determined by transient transfection of the p961-CAT construct into T47-D cells and uninduced and induced K562 cells. The p961-CAT construct directs CAT enzymatic activity with appropriate cell type specificity when transiently transfected into T47-D and K562 cells. When normalized for transfection efficiency, the p961-CAT construct produced 34-
fold greater enzymatic activity than CAT-structural sequences alone in T47-D cells, as shown in Fig. 5B. The a2961-CAT construct was inactive in uninduced and weakly active in induced K562 cells (Fig. 5C). Similar results were obtained in four separate experiments and a representative experiment shown.

To further characterize the 5′-flanking region required for either epithelial or megakaryocyte-specific promoter activity, 5′ deletion mutants of pα2961-CAT, pα2776-CAT (−776 to +109), pα2549-CAT (−549 to +109), pα292-CAT (−92 to +109), and pα30-CAT (−30 to +109) were constructed, as shown in Fig. 5A. The construct pα30-CAT, which retains only 30 bp 5′ to the transcription start site, failed to direct expression of the CAT gene in the epithelial cell line T47-D (Fig. 5B). The construct pα292-CAT, containing an additional 62 bp of the 5′-flanking region, was capable of directing activity in T47-D cells at 12% of the activity of the parent pα2961-CAT construct. The deletion mutants containing 549 or 776 bp of 5′-flanking sequence directed high-level activity in T47-D cells at 53% or greater than CAT-BASIC expression in this region. The pα2766-CAT construct, however, directs low-level activity in induced K562 cells. In contrast to the enhancer elements required for high-level expression in T47-D cells, the pα2549-CAT (-549 to +log), pα292-CAT (−92 to +log), and the parent construct directed high-level activity in T47-D cells at 100% of the activity of the parent pα2961-CAT construct (Fig. 5B). Based on these deletional analyses, enhancer activity necessary for high-level expression of the a2 gene in breast epithelial cells, as represented by the T47-D cell line, is located between bp −92 and −549 and in the distal 5′-end between bp −549 and −961. One estrogen half-site is located between −92 and −549, suggesting a possible role for estrogen in regulation of a2 expression. The diminished activity of the pα2549-CAT versus pα2961-CAT constructs suggests that additional enhancer elements are also located in the 412 bp between −549 and −961. Another estrogen receptor half-site as well as two 5′ regulatory sequences determined cell type- and differentiation-specific expression. The diminished activity of the pα2549-CAT construct may act as a silencer by altering chromatin conformation.

In summary, we have identified the promoter and enhancer regions of the a2 integrin gene which confer cell type-specific and differentiation-induced a2 gene expression. The identification of the a2 promoter will now allow dissection of the complex regulation of this integrin gene during normal differentiation, as well as carcinogenesis.

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