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GATA-1 binding to the α_V promoter negatively regulates expression of the integrin α_V subunit in human leukemic K562 cells✪

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Recently we observed that the transcription factors Sp1 and Sp3 bind to the CTCCTCCTC sequence located between positions –194 and –172 of the α_V promoter region and are directly involved in the regulation of transcriptional activity of the α_V gene in human umbilical vascular endothelial cells (HUVECs) (Czyz & Cierniewski, 1999, *Eur. J. Biochem.* 265, 638). In this report we provide evidence that the GATA-1 factor regulates α_V expression during differentiation of pluripotent K562 cells induced either by phorbol 12-myristate 13-acetate (PMA) or butyric acid (BA) through interaction with the GATA element in the α_V gene promoter. DNase I footprinting analysis revealed that region –413 to –408, covering the GATA binding site, was protected by nuclear extract from K562 cells. There was no protection of this region by HUVEC nuclear extract. Electrophoretic mobility shift assay (EMSA) analysis of nuclear extract of K562 cells treated with BA revealed an increase in GATA binding activity, which was associated with reduced α_V mRNA and α_V protein on the cell surface. Stimulation of K562 cells with PMA resulted in opposite effects: lower expression of GATA-1 was associated with increased levels of α_V. We conclude that the GATA-1 transcription factor specifically binds to the GATA element in the α_V gene promoter and negatively regulates α_V gene expression.

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Abbreviations: BA, butyric acid; EMSA, electrophoretic mobility shift assay; GATA-1, nuclear factor erythroid specific; GADPH, glyceraldehyde-3-phosphate dehydrogenase; HUVECs, human umbilical vein endothelial cells; PMA, phorbol myristate acetate; RT-PCR, reverse transcription-polymerase chain reaction; Sp1, Sp3, specificity protein 1, 3.

Interactions of integrin adhesion receptors with the extracellular matrix play an important role in the organisation of cells in differentiated organs as well as in cell motility, immune recognition, and cell aggregation [1–3]. Integrin expression appears to be highly regulated during many biological processes, but little is known about the molecular mechanisms that control their expression at the gene level. Usually, the level of expression of an integrin receptor is a consequence of tight regulation of one subunit mRNA combined with high steady-state expression of another subunit mRNA that maintains its constitutive availability [4, 5]. However, an increase in steady-state levels of both integrin subunit mRNAs has also been reported for example in endothelial and melanoma cells [4, 6, 7].

Vitronectin receptors consist of a group of integrins containing the α_V subunit in complex with several β subunits, including β_3, β_5, β_6 and β_8 [8, 9]. The synthesis of different $\alpha_V\beta$ receptors is modulated by growth factors, cytokines, and other biological substances [9–12]. Previous studies of $\alpha_V\beta$ protein synthesis have documented the existence of an intracellular pool of the α_V subunit in most cells, while the β subunits exhibit cell type and differentiation specific expression. A long-term alteration of α_V $\beta 3$ functions can be induced by modulation of gene expression, and regulatory regions in genes of both integrin subunits have been partially characterised [13, 14]. The α_V promoter lacks both the TATA and CAAT boxes, but contains four Sp1 binding sites, two Ets binding sites and one GATA binding site. In human endothelial cells and keratinocytes α_V integrin expression is controlled by a TCCTCCTCC motif in the promoter [15, 16].

Leukemic cell lines are very useful for studying the involvement of transcription factors in controlling hematopoietic cell differentiation. Upon exposure to appropriate stimuli pluripotent K562 cells can be induced to differentiate along a variety of pathways including erythroid, megakaryocytic, monocytic and lymphoid lineages. By employing phorbol myristate acetate (PMA) K562 cells can be induced to differentiate along the megakaryocytic pathway [17]. Low doses of butyric acid (0.5–1 mM) inhibit cell proliferation and induce erythroid differentiation [18]. The increased expression of GATA-1 and NF-E2 transcription factors is required during this process [19]. It has previously been reported that differentiation is associated with changes in cell surface expression of some integrins. For example, the megakaryocytic differentiation of K562 cells is accompanied by an increase in $\alpha 2\beta 1$ and αIIb$\beta 3$ proteins. Increased αIIb$\beta 3$ protein expression is due to higher steady-state levels of both αIIb and $\beta 3$ mRNAs resulting from transcriptional activation of both genes, whereas in the case of $\alpha 2\beta 1$ only $\alpha 2$ mRNA is changed [4]. Extensive research in the past few years has established the role for GATA-1, a transcription factor of the zinc finger family, in the process of differentiation [17, 19, 20].

In the present report we demonstrate that changes in α_V mRNA expression detected by RT-PCR and α_V antigen expression are associated with alteration of the GATA-1 mRNA expression, detected by RT-PCR, and with its DNA binding activity, shown by electrophoretic mobility shift assay (EMSA). This transcription factor appears to be involved in the regulation of α_V gene transcription in the human erythroleukemia cell line K562 during its differentiation.

MATERIALS AND METHODS

Reagents. [α^{32}P]dATP, 6000 Ci/mmol and T7 Sequenase v.2.0 were purchased from Amersham. Poly(dI-dC)·poly(dI-dC) and protein assay reagents were from Pharmacia and polyacrylamide gel chemicals were from BioRad. DNase I was from Boehringer Mannheim. All other reagents were from Sigma.

Plasmids. First, the fragment encoding the 5′-flanking region upstream from the translation initiation site of the α_V gene (–522 to +170) was transferred from the plasmid pαv940GH (kindly provided by Dr. J. Hawiger) into pBluescript II SK+ (Stratagene). The resulting plasmid pSKαvHX was used to prepare the DNA probe (–522 to –258) for footprinting analysis. Next, the 428-bp long, *Sac*I–*Xba*I fragment (–258 to +170) was cloned into pBluescript II SK+. Then, the *Sac*I–SacI fragment was cloned again into pBluescript II SK+ and the resulting plasmid pSKαv SX was used to prepare the DNA probe (–258 to +170) for footprinting analysis.

Cell culture and nuclear extract preparation. K562 cells, a human cell line with erythroleukemic characteristics, were grown in RPMI 1640 medium supplemented with 10% foetal calf serum, L-glutamine and penicillin/streptomycin. Cell viability was assessed by direct counting of trypan blue dye-excluding cells, and was above 95%. To study the influence of both agents, exponentially growing K562 cells were treated with either 5 nM phorbol 12-myristate 13-acetate (PMA) or with 1 mM butyric acid (BA). HUVECs were isolated and maintained in culture as described previously [15]. Crude nuclear extracts for footprinting experiments and electrophoretic mobility shift assays were prepared essentially as described by Dignam *et al.* [21], except that modified buffer C was used during nuclear extraction [22]. Protein concentrations were determined according to Bradford [23] using the BioRad protein assay reagents with bovine serum albumin (BSA) as a standard. To analyse cell differentiation towards erythrocytes, the percentage of haemoglobin-containing cells was determined by benzidine staining in cell suspensions.

In vitro *DNase I footprinting*. DNase I footprinting analysis was performed on end-labelled *Hin*dIII–SacI (–522 to –258) and *Hin*dIII–*Xba*I (–258 to +170) fragments from the α_V promoter as described previously [15]. Briefly, approximately 50 pmol aliquots of the probe were preincubated with either acetylated bovine serum albumin or nuclear extracts (30–150 μg of protein) in a total volume of 200 μl of reaction buffer (10 mM Hepes/KOH, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 10% glycerol, 0.01% Nonidet P-40, 2 μg/ml poly(dI-dC) · poly(dI-dC), for 10 min at room temperature. Then, DNase I was added and the reactions were stopped after 30 s with BSA or 1 min with nuclear extracts. The DNA was then precipitated and analysed on a 6% polyacrylamide/8 M urea gel along with Maxam–Gilbert sequencing reaction (G), then visualised on Kodak XAR-5 film at –20°C.

Electrophoretic mobility shift assays (EMSA). The oligonucleotide 5′-AAT TGG ACC GTA TCT CCC-3′ containing a GATA-1 binding site (underlined) was annealed with its complement, end-labelled with [α^{32}P]-dATP and Sequenase 2.0 (Amersham) and subsequently purified by 7% polyacrylamide non-denaturing gel electrophoresis in 0.5 × Tris/borate/EDTA. 5×10^4 c.p.m. were used for each EMSA. Binding reactions were performed in binding buffer (20 mM Hepes/KOH, pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol) in the presence of a non-specific competitor, poly(dI-dC) · poly(dI-dC), (Pharmacia). Crude nuclear extracts (15 μg) either from K562 cells or from HUVECs were incubated with 0.01 pmole of ^{32}P-labelled oligonucleotide duplex for 15 min at room temperature in a total volume of 20 μl. In competition experiments, either a 100-fold molar excess of an unlabelled double-stranded probe or a 200-fold molar excess of a double-stranded oligonucleotide with unrelated sequence: 5′-AAT TGA GGG AAA GAC CAA GA-3′ were used. All resulting protein–DNA complexes were separated from unbound probes by electrophoresis on 4% native polyacrylamide gels in 0.5 × Tris/borate/EDTA. Gels were vacuum-dried and autoradiographed on Kodak

XAR-5 film with intensifying screens at −20°C for 2 to 18 h.

Relative quantitative reverse transcription PCR assays of mRNA. To estimate the level of GATA-1 and α_V mRNAs during differentiation, mRNA from K562 cells treated with either PMA or BA was purified with the total RNA Prep Plus Kit (A&A Biotechnology). The first strand of cDNA was reverse transcribed according to the manufacturer's instructions using (dT)20 primer for 60 min at 30°C and stopped at 0°C. Then, mRNAs of α_V and GATA-1 were determined by relative quantitative RT-PCR using glyceraldehyde-3-phosphate dehydrogenase mRNA (GAPDH mRNA) as an internal standard. Assays employed the following primers: α_V mRNA: 5′-GATGGCAAAGGAGTACTTCCC-3′ and 5′-CGCTATCAATTCCTCACACTGC-3′; GATA-1 mRNA: 5′-GATCCTGCTCTGGTGTCCTCC-3′ and 5′-ACAGTTGAGCAATGGGTACACC-3′. RT-PCR assay conditions were as follows: 5 μl cDNA sample, 5 μl 10× Taq polymerase buffer (Epicentre Technologies), 2 mM $MgCl_2$, 1× PCR enhancer, dNTP mix, 25 pmol of each primer and 1.25 U of Taq DNA polymerase (Epicentre Technologies) in a 50 μl reaction volume. The samples were denatured at 95°C for 5 min, then cooled on ice and the enzyme was added. The reaction was performed for 25 to 30 extension cycles consisting of the following steps: 30 s denaturation at 94°C, 30 s annealing at 60°C, and 40 s polymerase extension at 72°C. Finally, each reaction was terminated with a 10 min elongation step at 72°C. To detect GADPH mRNA, the same protocol was used with oligodeoxyribonucleotide primers 5′-GAGAGATGATGACCCTTTTGGC-3′ and 5′-CCATCACCATCTTCCAGGAGCG-3′. The final products, cDNAs of α_V, GATA-1, and GAPDH were separated by electrophoresis in 7% polyacrylamide gels in Tris/acetate/EDTA buffer standardised against a 100 bp DNA Ladder (Promega). After staining with ethidium bromide bands were visualised by UV light, the results were recorded photographically, and analysed densitometrically using LKB Ultrascan XL Enhanced Laser Densitometr. Concentrations of mRNA for α_V and GATA-1 were normalised in each sample in relation to GAPDH mRNA.

FACS analysis. K562 cells (1×10^5) were treated with 5 nM PMA or 1 mM BA. Then the cells were suspended in DMEM containing 1% BSA and incubated with FITC-conjugated monoclonal antibodies against the α_V subunit (CHEMICON) at 4°C for 30 min in the dark. After double washing with 1% BSA in phosphate buffered saline (PBS), the cells were fixed by mixing with 1% paraformaldehyde in PBS and then analysed by fluorescence-activated cell sorting. The intensity of the staining by the antibody was measured with a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA). The data were analysed with PC Lysis II software.

RESULTS

DNA-protein interactions in the promoter of the α_V gene

To define DNA–protein interactions in the 5′ flanking region of the α_V gene, we first performed *in vitro* DNase I footprinting assays using fragments −522 to −258 and −258 to +170 of the promoter as DNA probes and nuclear extracts from either HUVECs or the human leukemic cell line K562. The major differences in the protection patterns were observed when the fragment encompassing the sequence from −522 to −258 was used. Protection was seen particularly well in the region containing the Sp1-Sp1-GATA-motif (Fig.1). The GATA binding site located at −415 to −408 was protected against DNase I digestion only by the nuclear extracts obtained from K562 cells (Fig. 1A, lane 1). HUVEC nuclear extracts had no effect on the DNase I footprinting pattern (Fig. 1A, lane 2).

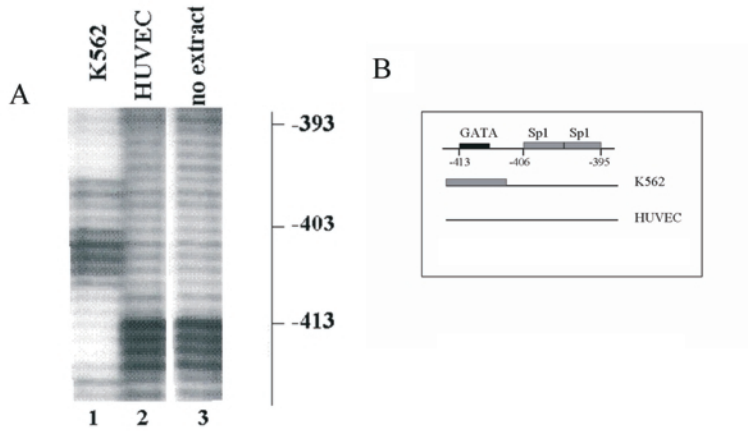

Figure 1. DNase I protection analysis of the *Hin*dIII–*Sac*I (–522 to –258) fragment of the α_V promoter.

A. DNase I digestion products obtained in the presence of nuclear extracts either from K562 cell line (lane 1) or from HUVEC (lane 2) were separated on a 6% sequencing gel. Lane 3 is the control without protein. In parallel, Maxam and Gilbert sequencing reaction products were separated (not shown). **B.** Sequence alignment of footprinted regions obtained with nuclear extracts from either K562 cell line or HUVECs. Two potential Sp1 and one GATA-1 binding sites are indicated and protected regions are boxed.

GATA binding activity is decreased in PMA-treated K562 cells but increased in BA-treated cells

Electrophoretic gel mobility shift assays were carried out to characterise the ability of the GATA motif at –410 to form a DNA–protein complex *in vitro* (Fig. 2). In these experiments, the radiolabelled oligonucleotide 5′-AAT TGG ACC GTA TCT CCC-3′ containing the GATA-1 binding motif and nuclear extracts from K562 cells differentiated upon stimulation with either PMA or butyric acid were used. The complex formed between the labelled probe and the proteins present in the nuclear extracts of the K562 cells migrated as a slow-moving band during electrophoresis. Its intensity depended upon culture conditions. A decreased amount of the complex was formed when the nuclear proteins were obtained from K562 cells treated with PMA (Fig. 2A, lane 4), whereas noticeably larger amounts of the labelled probe were shifted in the presence of nuclear extracts derived from K562 cells treated with butyric acid (Fig. 2A, lane 2). The complex of the labelled probe formed with nuclear proteins isolated from untreated K562 cells was used as a control (Fig. 2A, lane 1). In addition, nuclear extract from HUVECs was used (Fig. 2A, lane 3) and in this case only a small amount of the DNA probe was shifted. Since GATA-1 is not appreciably expressed in endothelial cells the slow-moving complex is probably due to the presence of the GATA-2 protein at a lower concentration and/or with lower affinity to the GATA binding site comparing to the affinity of the GATA-1 protein to the same binding site. All the complexes indicated appear to be specific for the GATA element as shown by the observation that addition of the cold probe to the binding reaction diminished the formation of the shifted band (Fig. 2B, lane 2), while addition of an oligonucleotide with unrelated sequence did not change the amount of the complex (Fig. 2B, lane 3).

Changes in GATA-1 and α_V expression during K562 differentiation

Based on the data from other laboratories suggesting that GATA-1 is involved in erythro-megakaryocytic differentiation [17, 19, 20] we focused on the role of GATA-1 in α_V expression. Expression of both GATA-1 and the α_V integrin subunit were examined at the level of mRNA in control and induced K562 cells. Treatment of the cells with butyric acid for 6 h resulted in a significant increase of the GATA-1 mRNA level that was accompanied by a decrease in the α_V mRNA (Fig. 3). On the other hand, stimulation of K562 with PMA caused an opposite effect, i.e. there was a re-

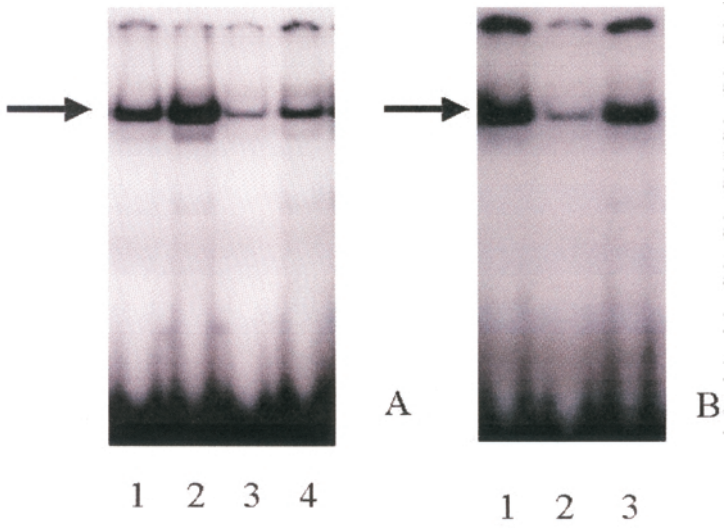

Figure 2. Electrophoretic mobility shift assay using labelled oligonucleotide containing GATA-1 and nuclear extracts from K562 cells.

A. Binding of nuclear proteins to the GATA-1 site was performed with 15 μg of protein from either untreated K562 cells (lane 1), BA-treated cells (lane 2), or PMA-treated cells (lane 4). In addition, nuclear extract from HUVECs was used (lane 3). **B**. Lanes 1–3 represent the GATA-1 binding activity detected in K562 cells treated with BA for 2 h. Homologous competition was performed in the presence of 100-fold excess of nonradioactive DNA probe (lane 2) and heterologous competition was done in the presence of 200-fold excess of oligonucleotide with unrelated sequence (lane 3). All samples were run on 4% native polyacrylamide gels. The specific DNA–protein complex is indicated by an arrow.

duced expression of the GATA-1 mRNA, but an enhanced level of the α_V mRNA. The same direction of changes was observed at the protein level measured by flow cytometry using monoclonal antibodies to α_V (Fig. 4). Upon treatment of K562 cells, PMA and butyric acid increased or reduced the cell surface expression of the α_V integrin, respectively. This indicates that α_V mRNA concentration and α_V protein surface exposure is down- or up-re-

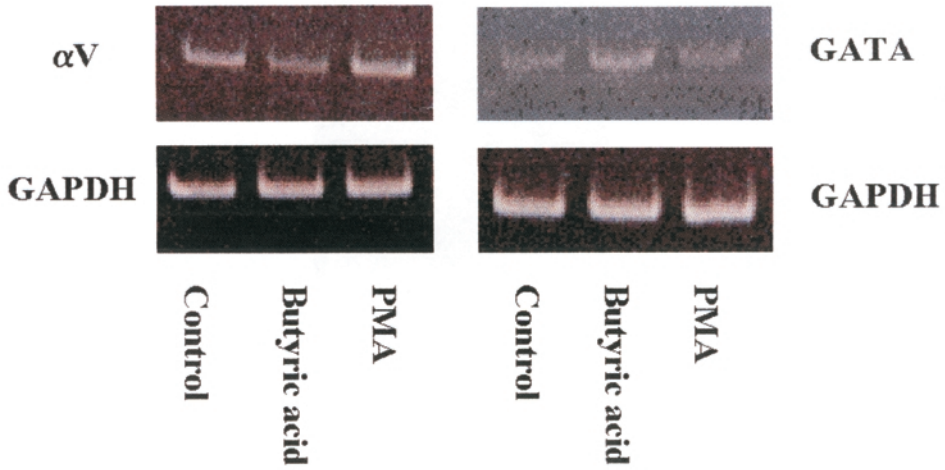

Figure 3. Effects of butyric acid and PMA on the expression of α_V and GATA mRNAs in K562 cells.

The cells were preincubated with 1 mM butyric acid or 5 nM PMA for 2 and 6 h, respectively, then changes in mRNAs of αV and GATA were estimated and compared to GAPDH mRNA levels. In parallel, basal expression of both mRNAs (Control) was estimated in cells that were not treated with these compounds. Treatment of cells with butyric acid reduced by 50% expression of αV mRNA and increased by two-fold GATA mRNA level. On other hand, PMA enhanced by two-fold expression of αV mRNA while inhibited by 30% GATA mRNA.

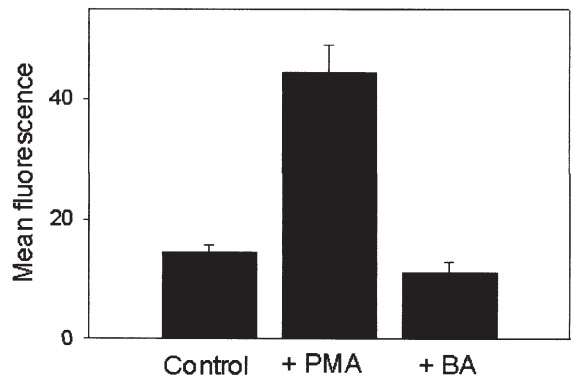

Figure 4. Effects of butyric acid and PMA on the expression of α_V antigen in K562 cells measured by flow cytometry.

K562 cells were treated with either 5 nM PMA or 1 mM BA as described in Materials and Methods. Then, the cells were suspended in DMEM containing 1% BSA and incubated with FITC-conjugated monoclonal antibodies against the α_V subunit (CHEMICON) at 4°C for 30 min in the dark. The intensity of the staining by the antibody was measured with FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, U.S.A.).

gulated when GATA-1 expression is modulated in K562 cells by butyric acid or PMA, respectively (Figs. 3 and 4).

DISCUSSION

Differentiation of pluripotential K562 cells, induced *in vitro* by different compounds, is associated with changes in the expression of several receptors belonging to the $\beta1$ and $\beta3$ integrin subfamilies [4]. Therefore, the K562 cell model can be used to characterise molecular events controlling the differentiation-dependent expression of these receptors. In this study, we analysed transcription factors which bind to the α_V promoter in K562 cells differentiated by treatment with PMA or butyric acid into megakaryocytic or erythroid cells, respectively. We used this model to examine a potential correlation between changes in GATA-1 and α_V expression. Our data indicate that the GATA-1 binding site is involved in negative regulation of the α_V integrin expression. This conclusion is based on the following observations: (a) Nuclear proteins of K562 cells, but not of HUVECs bind to the GATA element in the α_V gene promoter as shown by footprinting analysis. (b) Differentiation of K562 cells into erythroid cells is associated with a substantial decrease in expression of α_V detected both at the mRNA level and the protein concentration monitored at the cell surface. Under the same conditions, GATA expression is significantly enhanced as shown by much stronger signals given by this transcription factor during EMSA as well as after relative RT-PCR analysis of GATA mRNA. (c) The megakaryotic differentiation of K562 cells induced by PMA results in a dramatic increase in α_V expression on K562 cells detectable by flow cytometry. This is a consequence of the augmented steady-state level of α_V mRNA due to transcriptional upregulation of the α_V gene. GATA synthesis is decreased in these PMA-treated cells.

Interestingly, the GATA-1 site neighbouring two Sp1 binding sites can be found in the human α_V 5′ flanking region but is not present in the corresponding mouse promoter [24]. This raises the possibility that it may be dispensable for basal expression of the α_V protein but is used in selected cells under some physiological conditions. Thus, *via* the regulation of α_V level, the GATA element may regulate the expression of $\alpha_V\beta3$ receptors. We have previously reported that in human endothelial cells, α_V integrin expression is controlled by the TCCTCCTCC motif in the promoter, and the transcription factors Sp1 and Sp3 are the major nuclear proteins bound to this region [15]. Most recently, it has been found that Sp1 and Sp3 regulate β_5 gene expression in macrophages and osteoblast-like cells [25] and are responsible for myelomonocyte-specific phorbol ester downregulation of CD11d [26]. We have now observed that the GATA-1 site can also function as a negative regulator in the case of the α_V gene, in addition to other genes such as hu-

man ε-globin gene [27] and $^{A}\gamma$-globin gene [28]. Eukaryotic genes are regulated by a number of different *cis*-elements that are bound by different transcription factors. It is believed that differences in their combinatorial action form the basis for tissue- and developmental-specific gene regulation. In the case of the α_V gene the expression could be regulated at several levels. The CTC repeat would allow expression of the α_V gene in many cell types including HUVECs by binding different members of the Sp1 family with varying affinities. In addition, cluster sites such as Sp1-Sp1-GATA could determine a lower rate of transcription by binding the cell type specific factor GATA-1 during differentiation of hematopoietic progenitors. We cannot exclude that in certain tissues the transcriptional activity of the α_V gene depends on a coordination between Sp1 and GATA to provide tissue-specific expression levels. Such a mechanism was described for regulation of erythropoietin receptor in the human erythroid cell lines OCIM1 and K562 [29]. On the other hand, the same receptor in the human hepatoma cell lines Hep3B and $HepG_2$ is negatively regulated by GATA transcription factors [30] supporting the concept that changes in a transcription factor combination in the nucleus during differentiation or between particular tissues is one of the mechanisms by which eukaryotic genes are regulated.

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