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Analysis of the human CD34 gene promoter region in CD34 positive cell lines

Dissertation

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Abstract

CD34 is a cell surface glycoprotein expressed in stem and progenitor cells of hematopoiesis as well as in a subset of acute leukemia blasts. Mechanisms that control the human CD34 gene expression are largely unknown. Here I analyzed the role of the human protein nucleolin in regulation of the CD34 promoter region in CD34 positive cell lines KG-1a, KG-1 and NIH3T3. Cotransfection analyses using human CD34 promoter reporter constructs together with nucleolin expression plasmid were performed, showing that nucleolin is involved in the activation of the human CD34 promoter region. Furthermore, analyses of several mutant CD34 promoter constructs suggested an importance of two CD34 promoter region elements for the nucleolin-dependent activation. Moreover, increased amounts of the CD34 glycoprotein were detected by fluorescence-activated cell sorting on the surface of the CD34 positive cell line KG-1 after transfection with nucleolin expression plasmid.

Zusammenfassung

Yihua Du

Analyse des Promoterregions des humanen CD34-Gens in CD34 positiven Zellinien

CD34 ist ein Zelloberflächen-Glykoprotein, welches auf hämatopoietischen Stamm- und Vorläuferzellen sowie teilweise auf Zellen in akuter Leukämie exprimiert wird. Regulation der Expression des humanen CD34-Gens ist weitgehend unaufgeklärt. Ich analysierte die Rolle des humanen Proteins Nukleolin bei der Regulation des Promoterregions des humanen CD34-Gens in CD34 positiven Zellinien KG-1a, KG-1 und NIH3T3. Kotransfektionsversuche der CD34-Promoter Reporter-Konstrukte mit einem Nukleolin Expressions-Plasmid zeigten, dass Nukleolin bei der Aktivierung des Promoterregions des humanen CD34-Gens beteiligt ist. Weitere Analysen mit mehreren mutanten CD34-Promoter Reporter-Konstrukten wiesen darauf hin, dass zwei Elemente der Promoterregion des CD34-Gens eine wesentliche Rolle bei der nukleolinabhängigen Aktivierung spielen können. Darüber hinaus wurden nach Transfektion mit einem Nukleolin Expressions-Plasmid erhöhte Mengen des CD34-Glykoproteins an der Oberfläche CD34 positiver Zellen KG-1 mittels Durchflußzytometrie nachgewiesen.

Abbreviations

bp	base pair
cDNA	complementary DNA
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
EDTA	ethylenediamine tetraacetate
ES	embryonic stem
hnRNP C	heterogeneous nuclear ribonucleoprotein C
HSCs	hematopoietic stem cells
FCS	fetal calf serum
kb	kilobase pair
NCL	nucleolin
RBD	RNA binding domain
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNP	ribonucleoprotein
RRM	RNA recognition motif
rRNA	ribosomal RNA

Introduction

CD34 antigen is a glycoprotein , expressed on hematopoietic stem cells (HSCs) and progenitor cells (Civin, Strauss et al. 1984; Andrews, Singer et al. 1986); Simmons, Satterthwaite et al. 1992). It is frequently used as a marker for positive selection of human hematopoietic stem and progenitor cells in research and in transplantation, since bone marrow CD34 positive cells have the ability to reconstitute hematopoiesis in lethally irradiated baboons (Berenson, Andrews et al. 1988) and successfully reconstitute hematopoiesis by autologous and allogeneic transplantation in human (Civin, Trischmann et al. 1996; Link, Arseniev et al. 1996; Urbano-Ispizua, Rozman et al. 1997). Beside normal hematopoietic stem cells and progenitor cells, leukemia blasts from a subset of patients with acute myelogenous leukemia (AML) and most non-T, non-B acute lymphoblastic leukaemia (ALL) express CD34 (Tindle, Nichols et al. 1985; Batinic, Tindle et al. 1989). Moreover, CD34 has also been found to be expressed outside the hematopoietic system including in small vessel endothelial cells, a subset of malignant vascular tumors (Fina, Molgaard et al. 1990; Anthony and Ramani 1991; Nickoloff 1991; Traweek, Kandalaft et al. 1991; Fanburg-Smith, Michal et al. 1999), fibroblasts (Brown, Greaves et al. 1991; Greaves, Brown et al. 1992), stromal cells in gastric adenocarcinomas as well as lung adenocarcinomas (Nakayama, Enzan et al. 2001; Nakayama, Enzan et al. 2003), and dermatofibrosarcoma protuberans (Aiba, Tabata et al. 1992). It has been shown that both erythroid and myeloid differentiation from the embryonic stem cells (ES) were delayed in CD34-null mice. The colony-forming progenitors in bone marrow and spleen were notably fewer in adult CD34 deficient mouse (Cheng, Baumhueter et al. 1996). These data suggest that CD34 plays an important role in the formation of HSCs during both embryonic and adult

hematopoiesis. The CD34 molecule may be involved in leukocyte adhesion to endothelial cells or bone marrow stromal cells, perhaps severing as a ligand of stromal lectins (Simmons and Torok-Storb 1991; Baumheter, Singer et al. 1993). CD34 may also involved in interaction with cell-cell adhesion between homotypic CD34 positive cell lines (Traore and Hirn 1994) . However, the function of the CD34 molecule is largely unknown.

The expression pattern of the CD34 gene between human and mouse is conserved. The murine CD34 gene encodes a closely homologous protein expressed on hematopoietic progenitor cells and the embryonic fibroblast suggesting a conserved function of CD34. The human CD34 gene and the murine CD34 gene have been cloned (Brown, Greaves et al. 1991; He, Antao et al. 1992; Simmons, Satterthwaite et al. 1992). The analysis has shown that the homologues between human and murine CD34 are very extensive (Brown, Greaves et al. 1991; He, Antao et al. 1992). A homology between human and mouse was also identified in the 5' flanking regions of CD34 genes (Brown, Greaves et al. 1991; He, Antao et al. 1992; Simmons, Satterthwaite et al. 1992). The 5' flanking regions of both the human CD34 and the murine CD34 genes revealed the absence of typical promoter motifs, TATA box and/or CAAT box (Brown, Greaves et al. 1991; He, Antao et al. 1992; Simmons, Satterthwaite et al. 1992). Several potential DNA binding sites for transcriptional regulatory proteins, including c-Myb, c-Myc, and six Ets-like binding motifs have been identified depending on the DNA sequence (Burn, Satterthwaite et al. 1992; He, Antao et al. 1992). However, only some of the targets have been identified in the regulation of human or murine CD34 gene expression. Ets-2 and c-Myb transactivate the CD34 promoter independently (Melotti and Calabretta 1994; Melotti, Ku et al. 1994), a nuclear factor Y (NFY) positively regulates the CD34 gene in transient transfection experiments (Radomska, Satterthwaite et al. 1999), whereas a zinc finger protein MZF-1 inhibits chloramphenicol

acetyltransferase activity driven by the CD34 promoter region have been reported (Morris, Rauscher et al. 1995; Perrotti, Melotti et al. 1995). Despite extensive research thus far, little is known about the regulation of the expression and the function of the CD34 molecule.

Nucleolin is a major nucleolar protein. It has been suggested that nucleolin is necessary for cellular protein synthesis, as it is probably involved in the biosynthesis of ribosomes (Srivastava and Pollard 1999). Subsequent studies have shown that nucleolin was a multifunctional nucleolar phosphoprotein and involved in many metabolic processes, such as cell growth, cell proliferation (Caizergues-Ferrer, Mariottini et al. 1989; Srivastava and Pollard 1999) and cell death which have been highlighted in several reviews (Tuteja and Tuteja 1998; Ginisty, Sicard et al. 1999; Srivastava and Pollard 1999). It has been suggested that nucleolin is preferentially expressed in proliferating and less in differentiating cells (Srivastava and Pollard 1999). Nucleolin is highly expressed in embryogenesis (Schneider and Issinger 1989; Maridor, Krek et al. 1990; Messmer and Dreyer 1993; Baran, Mercier et al. 1997) and exceedingly low in several adult tissues (Mercier et al. 1997) implicating it in functions during embryogenesis and development. More recently nucleolin has been described as a cell surface marker of angiogenic endothelial cells within the vasculature and a new L-selectin ligand in human cells (Harms, Kraft et al. 2001; Christian, Pilch et al. 2003). Human nucleolin cDNA has been cloned (Srivastava, Fleming et al. 1989). The protein sequence analysis showed that nucleolin consists of 707-amino acid. Numerous studies have demonstrated that nucleolin is a multifunctional protein, which contains three different functional domains. (i) The acidic histone-like N terminal domain has been implicated in interaction with chromatin, involved in ribosome assembly and preribosomal RNA transcription (Lapeyre, Bourbon et al. 1987; Erard, Belenguer et al. 1988). (ii) The central region of nucleolin containing four

RNA binding domains (RBDs) is involved in specific interactions with RNA (Bourbon, Lapeyre et al. 1988; Srivastava, Fleming et al. 1989). It has identified the nucleolin binding specifically to the site in RNA, which has a consensus sequence (U/G)CCCG(A/G) in the context with a short hairpin loop structure (Ghisolfi-Nieto, Joseph et al. 1996; Bouvet, Jain et al. 1997; Finger, Trantirek et al. 2003; Johansson, Finger et al. 2004). (iii) The C terminal domain of nucleolin, which is rich in arginine and glycine, called glycine arginine rich domain (GAR domain). There are several Arg-Gly-Gly motifs interspersed with a few phenylalanine residues in GAR domain, mainly involved in pre-rRNA packaging, RNA and/or protein interactions (Messmer and Dreyer 1993; Bouvet, Diaz et al. 1998). These observations suggest an essential role for the GAR domain in mediating interaction of nucleolin with targeting of some ribosomal proteins and might be also involved in the RNA-binding (Heine, Rankin et al. 1993; Najbauer, Johnson et al. 1993).

Nucleolin is a DNA and RNA binding protein (Williams, Brys et al. 1992; Williams, Hanakahi et al. 1993; Dickinson and Kohwi-Shigematsu 1995; Hanakahi, Dempsey et al. 1997; Ying, Proost et al. 2000). Previous studies demonstrated that nucleolin and a heterogeneous nuclear ribonucleoprotein C (hnRNP C) specifically bind to a 29-base element within the 3'-untranslated region of amyloid precursor protein mRNA (Zaidi and Malter 1995). Gene targeting studies have demonstrated that nucleolin is one of the components of the transcription factor LR1 in B cells (Brys and Maizels 1994; Hanakahi, Dempsey et al. 1997; Hanakahi and Maizels 2000). LR1 binds to the μ gene enhancer (Eμ) in immunoglobulin heavy chain μ locus as a transcriptional activator has also been reported (Hanakahi and Maizels 2000). Recently, our laboratory has demonstrated that nucleolin is an activator of the transcription for the human papillomavirus type 18 oncogenes (Grinstein, Wernet et al. 2002). Thus, a role of nucleolin in

transcriptional and post-transcriptional regulation of gene expression has been established.

In the present thesis, I analyzed the possibility that nucleolin may participate in modulating the activity of the human CD34 gene promoter region. Cotransfection experiments in several CD34 positive cell lines were preformed. The data revealed an involvement of nucleolin in the activation of the CD34 promoter region in CD34 positive cell lines under study, which occurs via at least one CD34 promoter element. In addition, increased amounts of the CD34 glycoprotein were detected by fluorescence-activated cell sorting analysis on the surface of the CD34 positive cell line KG-1 after transfection with the human nucleolin expression plasmid.

Materials and Methods

Cell Lines culture conditions

All cell lines for this study were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig).

NIH 3T3 (DSMZ No. ACC 59), murine embryo fibroblasts (Jainchill, Aaronson et al. 1969) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.), supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mmol/L L-glutamine at 37 °C with 5% CO2.

KG-1 (DSMZ No. ACC 14), a human acute myeloid leukemia cell line (Koeffler and Golde 1978) were grown in suspension in RPMI 1640 (Gibco, BRL, Grand Island, NY) medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mmol/L L-glutamine at 37 °C with 5% CO2.

KG-1a (DSMZ No. ACC 421), a human myelogenous leukemia cell line (Koeffler, Bar-Eli et al. 1981) were grown in RPMI 1604 medium (Gibco, BRL, Grand Island, NY) supplemented with 20% heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mmol/L L-glutamine at 37 °C with 5% CO2.

Construction the expression plasmid and reporter plasmids

Nucleolin in eukaryotic expression vector pCMVTag2B:

The full-length human nucleolin cDNA cloned in the pMAM plasmid was obtained from Dr. Grinstein. The plasmid was digested with *Bam*HI and *Xho*I restriction enzymes and the fragment containing nucleolin cDNA was

collected with a Gel Extraction Kit (Qiagen), and inserted into the eukaryotic expression vector pCMVTag2B (Strategene). The resulting expression plasmid is referred to as pCMV/nucleolin. The sequence of the construct was verified by DNA sequencing analysis.

Construction 5'flanking region of the human CD34 gene luciferase reporter Constructs:

The constructs contain 5' flanking region of the human CD34 gene (Figure 1B) used in this study are depicted in Figure 2. These constructs were inserted into the promoterless luciferase reporter vector pGL3Basic (Promega). The construct containing the nucleotide sequence from –666 to +174 of the human CD34 promoter region inserted into the pGL3Basic vector was obtained from Dr. Grinstein, and is referred to pGL3/CD34^{wt}-luc throughout the text. The construct pGL3/CD34^{wt}-luc was sequenced, showing in Figure 1B that is the same as the published human CD34 gene sequence (He, Antao et al. 1992).

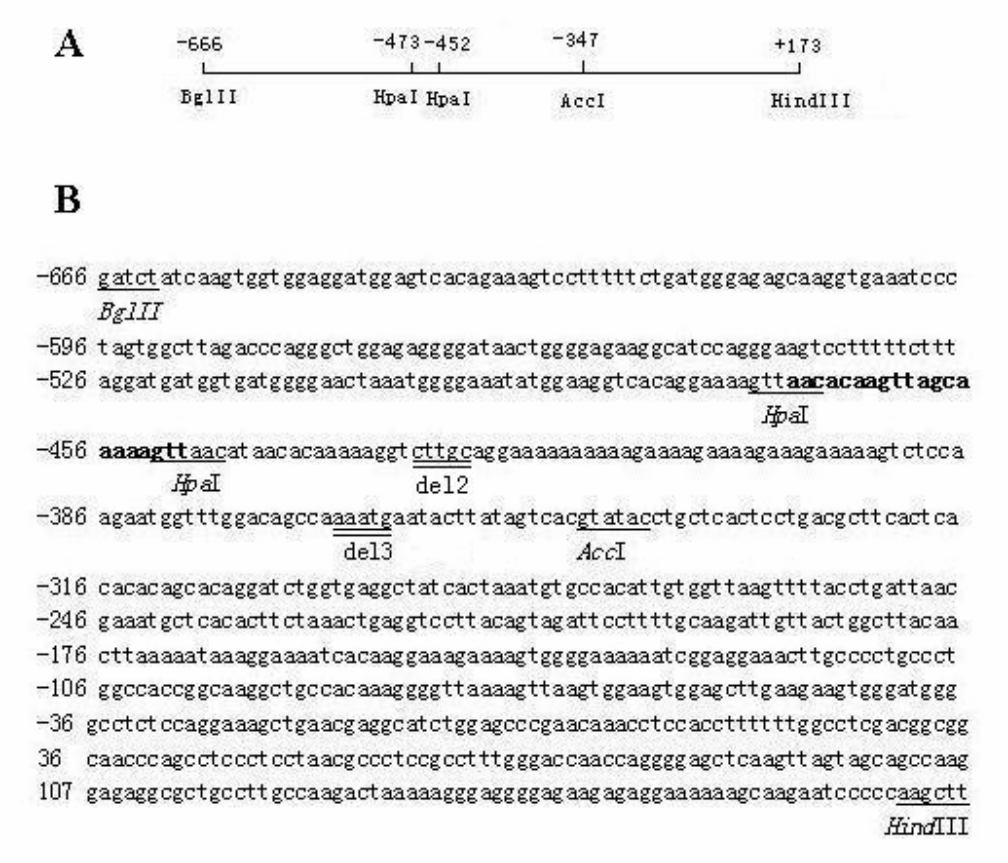

Figure 1. Restriction enzyme map and sequence of the 5'-flanking region of the human CD34 gene. (A) The schematic restriction map for the subcloned human CD34 promoter region. (B) The alignments of DNA sequence of the cloned CD34 promoter region. The restriction sites (*Bgl*II, *Hpa* I, *Acc* I, and *Hind* III) used in the preparation of the constructs in this experiment are indicated and underlined. The nucleotide position of the synthesized 21bp (between the two original *Hpa*I sites in human CD34 promoter region) is indicated in boldface. The sequences deleted in mutant deviations of the 5' flanking region are double underlined.

Construction of the site-deletion human CD34 promoter luciferase reporter constructs:

Following here is the description of the construction of the human CD34 promoter region mutants used in this study. The construct containing the sequence from –666 nt. to +174 nt. of the human CD34 promoter region in the pCR2.1TOPO vector (Invitrogen) was obtained from Dr. Grinstein and employed as a template for the construction of the deletion mutants. All CD34 promoter region fragments with different deletions were generated by PCR using primer pairs with site-deletions (see table 1 for the primers sequence information). The sequence information and the positions of the deletions in this study are shown in Figure 1B. Del 2 (deletion from –431 nt. to –427 nt.) and Del 3 (deletion from –367 nt. to –363 nt.).

Table 1. Primers used for amplification of CD34 promoter region with different deletions and mutation.

Construct	Forward primer with HpaI site	Reverse primer with AccI site
Del 2	5'-AACATA ACACAAAAAGGTAGGAAAAAAA-3’	5'-GAGCAGGTATACGTGACTATAAG-3'
Del 3	5'-AACACAAGTTAGCAAAAAGTT-3'	5'-ATTATAGTATACGTGACTATAAGTATTTGGCTGTC –3'
Synthesis 21bp	5'-AACACAAGTTAGCAAAAAGTT-3'	5'-AACTTTTTGCTAACTTGTGTT-3

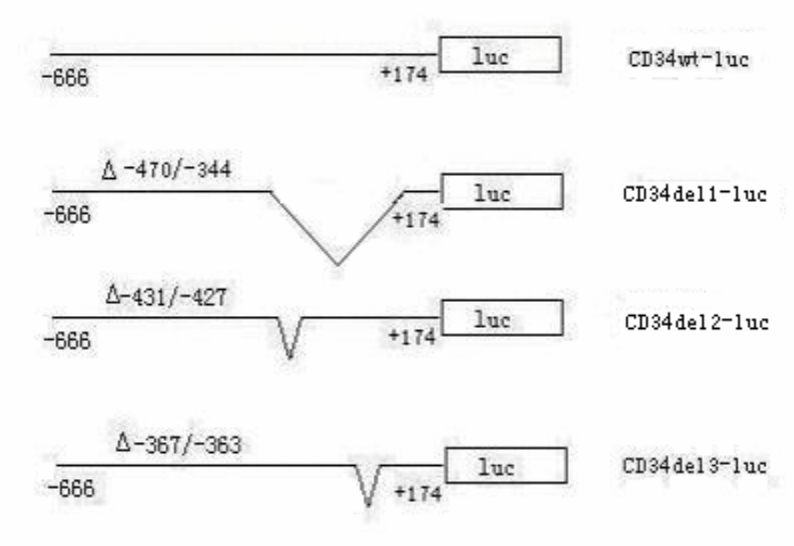

Figure 2. CD34 gene reporter constructs used in this study. The firefly luciferase reporter gene is shown as a hollow rectangle at the right-hand side of each construct. Thin black lines extending horizontally toward the left from the luciferase gene represent the promoter region of the human CD34 gene. From the top to the bottom are in turn with: pGL3/CD34^{wt}-luc, pGL3/CD34^{del1}-luc, pGL3/CD34^{del2}-luc, and pGL3/CD34^{del3}-luc plasmid. Numbers below each construct indicate nucleotide (nt.) position. Numbers above each construct indicate nucleotide with respect to the deletion sites. Constructs are not drawn to the same scale.

To construct human a CD34 promoter region with deletion 3, a two-step subcloning is required for the plasmid pGL3/CD34^{del3}-luc. The first step is to generate the plasmids containing the site-deletion 3 into vector pCR2.1TOPO to get plasmid pCD34^{del3}/TOPO (see figures 1B and 2). The forward primers 5’ contained HpaI restriction site and the reverse primer 5’ contained AccI restriction site. Because HpaI is a restriction site with a blunt end, the forward primer 5' ends were designed with a part of HpaI site, After PCR amplification, the amplified DNA fragment were digested with AccI only. The plasmid pCD34^{wt}/TOPO was double digested with HpaI-AccI.

The HpaI-AccI fragment of pCD34^{wt}/TOPO (4.6Kb) were collected and ligated with AccI digested PCR fragment to generate the pCD34^{del3}/TOPO. The PCR generated plasmid pCD34^{del3}/TOPO was verified by DNA sequence before the second step subcloning. The second step is the sub-cloning the CD34 promoter region with the site-deletion 3 into the upstream of luciferase reporter vector pGL3Basic. The plasmid pCD34^{del3}/TOPO was digested with BglII-HindIII. The fragment was ligated with BglII-HindIII digested pGL3Basic to generate pGL3/CD34^{del3}-luc (deletion from -367 nt. to -363 nt. , deletion sequence is AAATG).

In the 1st step of subcloning, when selecting the clones, I have isolated one clone with a long fragment deletion (deletion from *Hpa*I site to *Acc*I site) in the ligated plasmid. That is the deletion 1 construct pCD34^{del1}/TOPO. I used this plasmid pCD34^{del1}/TOPO, which was digested with *Bgl*II-*Hind*III and sub-cloned it into *Bgl*II-*Hind*III digested pGL3Basic to generate pGL3/CD34^{del1}-luc (deletion from –470 nt. to –344 nt.)(See Figures 1A, 1B and 2).

To generate the plasmid pGL3/CD34^{del2}-luc, a three-step subcloning is needed. The first step: The fragment with site deletion 2 was amplified by PCR. The PCR fragment was digested with AccI only, and ligated with 4.6 Kb HpaI-AccI digested pCD34^{wt}/TOPO; The 2nd step: digested this plasmid with HpaI, and insertion into the synthesized 21bp fragment to generate the plasmid pCD34^{del2}/TOPO (see Figure 1B); The 3rd step: The plasmid pCD34^{del2}/TOPO was digested with BglII-HindIII. The fragment was sub-cloned into BglII-HindIII digested pGL3Basic to generate pGL3/CD34^{del2}-luc (deletion from –431 nt. to -427 nt., deletion sequence is CTTGC). All the plasmids were prepared with Endotoxic Free Plasmid Maxi kit (Qiagen) for cell transfection assays.

Transfection experiments.

Transient transfection of NIH 3T3 cells

NIH 3T3 (murine embryonic fibroblast) cells were transfected using Polyfect transfection kit (Qiagen) following the manufacturer's protocol. NIH 3T3 cells were seeded in 6 well culture plates at 15% confluence 16-18 hours before transfection. The cells were transiently transfected with 1 µg of reporter constructs (pGL3/CD34^{wt}-luc, pGL3/CD34^{del1}-luc, pGL3/CD34^{del2}-luc, pGL3/CD34^{del3}-luc, or the promoterless pGL3Basic vector) and 0.5 µg of expression plasmid pCMV/nucleolin or the empty vector pCMVTag2B, respectively, using 10 µL of Polyfect transfection reagent per well (Qiagen). The promoterless pGL3Basic luciferase reporter vector was used in all experiments as a baseline control. After co-transfections, the cells were incubated for 48 h at 37°C, 5% CO_2 before assaying luciferase activity.

Nucleolin titration in NIH 3T3 cells.

To analyze the activity of the pGL3/CD34^{wt}-luc reporter construct in the presence of different amounts of the nucleolin expression plasmid, NIH 3T3 cells were cotransfected using 1 µg of reporter construct pGL3/CD34^{wt}-luc with 0.25, 0.4, or 0.55 µg of the effector plasmid pCMV/nucleolin, respectively. pGL3Basic vector was used as a baseline control. The transfected cells were incubated 48 hours at 37°C in a 5% CO_2 atmosphere for subsequent luciferase assays.

Stable transfection of NIH 3T3 and KG-1 cells.

NIH3T3 cells were transfected with the human nucleolin expression plasmid (pCMV/nucleolin) or the empty expression vector (pCMVTag2B) using Polyfect transfection reagent (Qiagen) according to the manufacturer’s

instructions. 48 hours after transfection, cells were cultured in Dulbecco's modified Eagle's medium containing 200 µg/ml G418 and 10% FCS for 2 weeks. The G418-resistant cell lines NCL-3T3 and CMV-3T3 were subsequently grown in DMEM medium containing 100 µg/ml G418 and 10% heat-inactivated fetal calf serum.

For assays of CD34 promoter region activity, the two cell lines NCL-3T3 and CMV-3T3 were transiently transfected with 1 µg of the pGL3/CD34^{wt}-luc reporter construct or pGL3Basic respectively, using 10 µl of Polyfect transfection reagent (Qiagen) per well, and processed further as described above.

Transient transfection of KG-1 and KG-1a cells.

KG-1 or KG-1a cells were transfected using DMRIE-C transfection reagent according to the manufacturer's protocol (Invitrogen). Briefly, KG-1 or KG-1a cells were grown to a density of 1-1.5x10^6 cells/ml in RPMI 1640 medium prior to transfection. Cells were cotransfected in triplicates in 6 well plates, using 3 µg of a luciferase reporter construct (pGL3/CD34^{wt}-luc, pGL3/CD34^{del2}-luc, or pGL3/CD34^{del3}-luc) either with 0.5 µg of an expression plasmid pCMV/nucleolin or with 0.5 µg of expression vector pCMVTag2B, respectively. The total amount of DNA pre transfection was made equal by the addition of an appropriate amount of the empty vector pCMVTag2B.

6 µl of DMRIE-C reagent diluted in 500 µl of Opti-MEM I medium (Invitrogen) was added pre well. The appropriate amounts of the plasmids DNA indicated above were diluted with 500 µl of Opti-MEM I medium. The diluted DMRIE-C reagent was mixed with the diluted plasmid DNA and incubated for 30 minutes at room temperature. The KG-1 or KG-1a cells were pelleted, washed once with no serum, no antibiotic RPMI 1640

medium, and resuspended in Opti-MEM I medium to adjust the cell confluence to $2x10^6$ cells in 200 µl Opti-MEM I medium. The $2x10^6$ cells in 200 µl Opti-MEM I medium were added per well. After transfection, cells were incubated for 5 hours at 37°C in 5% CO_2. Then 2 ml of growth media RPMI 1640 containing 20% FCS was added per well, and further incubated at 37°C with 5% CO_2 atmosphere for 48 hours.

Nucleolin titration in KG-1a cells.

To analyze the activity of the pGL3/CD34^{wt}-luc reporter construct in the presence of different amounts of pCMV/nucleolin plasmid, KG-1a cells were cotransfected with 3 µg of the pGL3/CD34^{wt}-luc construct and 0.1 µg, 0.3 µg, or 0.5 µg of the pCMV/nucleolin plasmid, respectively. 3 µg of pGL3Basic vector with 0.5 µg of pCMVtag2B was used as a baseline control. The total amount of the DNA was kept consistent by adding pUC18 plasmid to adjust to 3.5 µg/well. The transfection methods were the same as described above. The transfected cells were incubated for 48 hours at 37°C in a 5% CO_2 atmosphere for luciferase assays.

Luciferase Reporter Assays.

NIH 3T3 cells were harvested 48 hours after transfection. The cells were trypsinized with trypsine-EDTA for 2 minutes and neutralized with ice-cold phosphate-buffered saline (PBS). The cell pellet was obtained by gentle centrifugation. Cells were gently washed once with cold PBS and lysed in 150 µl of lysis buffer supplied together with the luciferase assay kit (Roche) for 5 min at room temperature. According to the manufacturer's protocol, 50 µl of cell lysates were mixed together with 50 µl of the luciferase assay reagent (Roche), and the light emission was measured immediately with a MiniLumat LB 9507 luminometer (Berthold, Wildbad, Germany). Protein concentration in the cellular lysates was quantitated by

the Bradford method with the Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA). Luciferase activity was normalized to protein concentration and compared to that of pGL3Basic. All experiments were repeated at least three times with at least five independent DNA preparations.

KG-1 and KG-1a cells were harvested 48 h after transfection. Cells were washed twice with ice-cold PBS and transferred to microcentrifuge tubes. The cell pellet was lysed in 150 µl of 1 × Lysis Buffer (Roche), as described above. The lysates were clarified by centrifugation at the maximum (15,000 rpm) for 2 minutes. 100 µl of the cell lysates were used for measurements of light emission as described above, using a MiniLumat LB 9507 luminometer (EG & G Berthold, Bad Wildbad, Germany). Luciferase activity was normalized to protein concentration and compared to that of pGL3Basic. Individual cotransfections were repeated at least three times with three independent preparations.

Generation of the stable nucleolin transfected KG-1 cells and Analysis of the human CD34 molecule by flow cytometry.

KG-1 cells were transfected with the pCMV/nucleolin plasmid or the pCMVTag2B plasmid using DMRIE-C transfection reagent (Invitrogen). Briefly, KG-1 cells were grown to a density of $1x10^6$ cells/ml in RPMI 1640 medium before transfection. Cells were transfected in 5ml flasks, using 15 µg of the pCMV/nucleolin plasmid or pCMVTag2B vector. Mix 24 µl of DMRIE-C reagent in 3 ml of Opti-MEM I medium (Invitrogen), incubate at room temperature for 30 min. dilute 15 µg of expression plasmid pCMV/nucleolin with 1 ml of Opti-MEM I medium; The control was using 15 µg of expression vector pCMVTag2B in 1 ml of Opti-MEM I medium. Mock control contained no DNA. Mix the diluted DMRIE-C reagent with the diluted plasmids DNA and incubate for 30 minutes at room temperature.

The KG-1 cells were pelleted, washed once with no serum, no antibiotic RPMI 1640 medium, and resuspended in Opti-MEM I medium to adjust the cell confluent as $8x10^6$ cells in 800 µl Opti-MEM I medium. Add the $8x10^6$ cells in each flask. After transfection, cells were incubated for 5 h at 37°C in 5% CO_2. Then, add 8 ml of growth media RPMI 1640 containing 15% FCS in each flasks. Incubate the transfected cells at 37°C in 5% CO_2 for 48 hours. 48 hours post transfection, Cells were cultured in RPMI 1640 medium containing 10%FCS and 200 µg/mL G418 for three weeks until the mock has only 5% or less living cells. After selection, the two G418-resistant cell lines were maintained in the RPMI 1640 medium containing 10%FCS and 100 µg/ml G418. The resulting cell lines NCL-KG1 and CMV-KG1 were used for FACS analysis of the presence of the CD34 glycoprotein at the cell surface.

Flow cytometry of phycoerythrin (PE)-conjugated anti-human CD34 mouse monoclonal antibody.

The stably transfected NCL-KG1 and CMV-KG1 cells were harvested by gentle centrifugation, and washed with ice-cold PBS. Cells were suspended in 50 µl of PBS containing 0.5% goat serum, and 5 µl of phycoerythrin (PE)-conjugated anti-human CD34 mouse monoclonal antibody were incubated with each sample for 30 minutes on ice. Subsequently, 100 µl of Intraprep 1 reagent was added, followed by incubation for 20 minutes on ice. Cells were washed with 1.5 mL PBS for flow cytometry; FACSCalibur device (Becton Dickinson) was used. The data were analyzed with CellQuest Software.

Results

Activation of the human CD34 promoter region by nucleolin.

It has been demonstrated previously that nucleolin acts as an activator of the human papillomavirus type 18 oncogene transcription (Grinstein, Wernet et al. 2002). A preliminary analysis has shown that a 0.8 kb fragment of the human CD34 promoter region (from –666 nt. to +174 nt.) was active in NIH 3T3 cells, a murine embryonic fibroblast cell line (Figure 3, column 2). The activity was approximately 3 fold in comparison with the promoterless pGL3Basic vector (Figure 3, column 6). The effect of human nucleolin on the CD34 promoter region activity was then analyzed by means of cotransfection assays.

The wild type human CD34 reporter construct, pGL3/CD34^{wt}-luc was cotransfected either with the full-length human nucleolin expression plasmid or with pCMVTag2B (the empty expression vector), respectively. The relative luciferase activity of pGL3/CD34^{wt}-luc (Figure 3, column 1) to that of pGL3Basic (Figure 3, column 5) was averagely 8-fold increased when the NIH 3T3 cells were cotransfected with nucleolin expression plasmid (Figure 3, compare column 1 to column 5). These results indicate an involvement of nucleolin in activation of the human CD34 promoter region in a CD34 positive cell line. To show that this effect depends on a distinct domain within the human CD34 promoter region, we used the construct pGL3/CD34^{del1}-luc, which has a 127 nt. deletion within the promoter region (deletion from –470 nt. to –344 nt.) for the transient transfection assays. NIH 3T3 cells were cotransfected with pGL3/CD34^{del1}-luc construct either with the human nucleolin expression plasmid pCMV/nucleolin (Figure 3, column 3) or with the empty pCMVTag2B vector (Figure 3, column 4). The cells were also cotransfected with luciferase reporter vector pGL3Basic and

the expression vector pCMVTag2B as the baseline control (Figure 3, column 6). Comparison of the transcriptional activity of the deletion construct pGL3/CD34^{del1}-luc, which lacking the 127 nt. sequences within human CD34 promoter region with that of the pGL3Basic vector, we found the relative luciferase activity of pGL3/CD34^{del1}-luc (Figure 3, column 4) was not higher than that of the empty luciferase report vector pGL3Basic (Figure 3, column 6). The cotransfection of the pGL3/CD34^{del1}-luc construct with the nucleolin expression plasmid showed quite little increasing in luciferase activity compare to the transfection of pGL3/CD34^{del1}-luc (Figure 3, compare column 3 to column 4). These data suggest that the fragment from –470 nt. to -344 nt. in the 5' flanking region of the human CD34 gene is necessary for the promoter activity in NIH 3T3 cells.

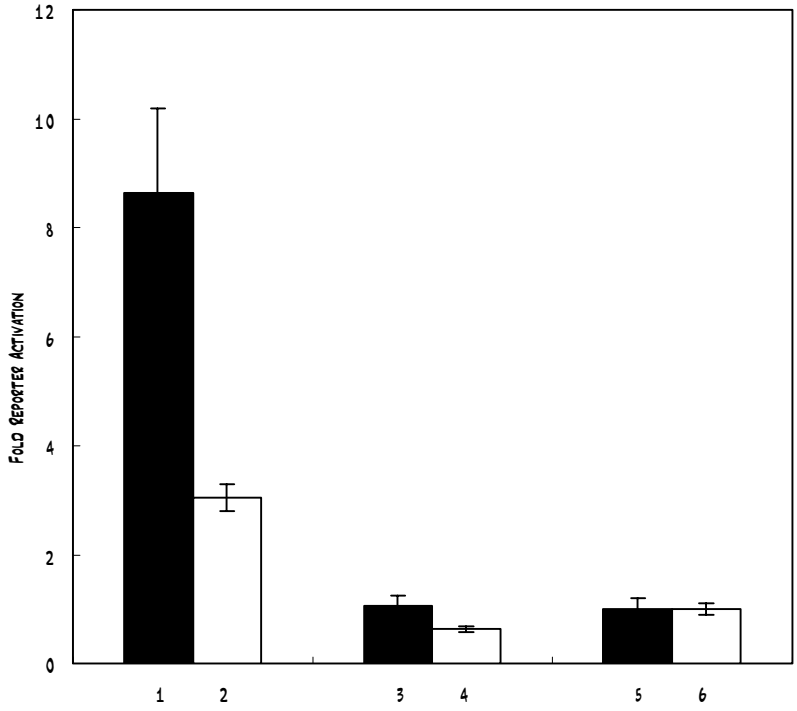

Figure 3. Nucleolin dependent activation of the human CD34 promoter region in NIH 3T3 cells. The CD34 positive murine NIH 3T3 cells were transfected with either pGL3/CD34^{wt}-luc or pGL3/CD34^{del1}-luc depicted in figure 2. *White bars* (□), luciferase activity of the human CD34 constructs pGL3/CD34^{wt}-luc (column

2) or pGL3/CD34^{del1}-luc (column 4) were cotransfected with empty expression vector pCMVTag2B, respectively. The cells were cotransfected with pGL3Basic vector and pCMVtag2B vector as the baseline control (column 6). *Black bars* (■), cotransfection of human CD34 constructs pGL3/CD34^{wt}-luc (column 1) or pGL3/CD34^{del1}-luc (column 3) with human nucleolin expression plasmid, respectively. Cotransfection of the promoterless pGL3Basic vector with nucleolin expression plasmid was used as the baseline control (column 5). 48 hours post-transfection, the cells were harvested. Luciferase activities were normalized to the protein concentration. Fold activation to that of the pGL3Basic is plotted on the ordinates. (bars, ± SE.) The values represent five independent samples. Error bars indicate the standard deviations. The transfection experiments were preformed three times.

Activity of the human CD34 promoter region in NIH 3T3 cells is dependent on the amounts of nucleolin

To further investigate the relationship between nucleolin and the activity of human CD34 promoter region, the activity of the pGL3/CD34^{wt}-luc construct was measured in the presence of different amounts of nucleolin expression plasmid. The effect of nucleolin on the human CD34 promoter activity was dose-dependent (Figure 4, columns 2, 3 and 4). These data further indicated the involvement of nucleolin in activation of the human CD34 promoter region.

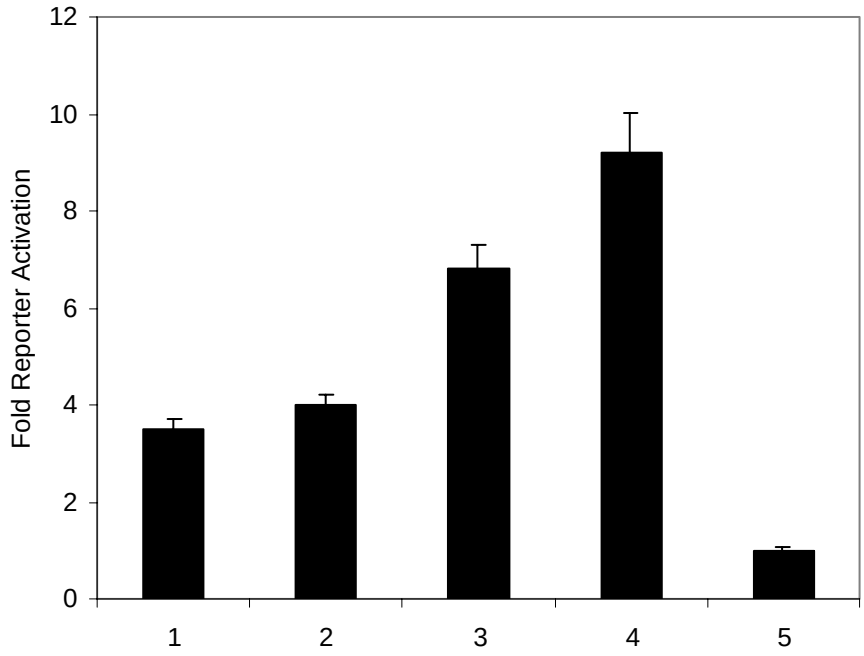

Figure 4. Activation of the human CD34 promoter region by nucleolin in NIH 3T3 cells. NIH 3T3 cells were cotransfected with 1 µg of pGL3/CD34^{wt}-luc and different amounts of nucleolin expression plasmid, respectively. The amounts of nucleolin expression plasmid used in the cotransfection assays were 0.25 µg (column 2), 0.4 µg (column 3), and 0.55 µg (column 4), respectively. Cotransfection of pGL3Basic vector with the empty expression vector pCMVtag2B was used as the baseline (column 5). The cells were also cotransfected with pGL3/CD34^{wt}-luc and pCMVtag2B vector (column 1). Luciferase activity was measured 48 hours after transfection. The activity of luciferase was normalized to protein concentration. The values are average of transfections performed in triplicate. Error bars indicate the standard deviations.

Analysis of the activity of deletion mutants of the human CD34 promoter in NIH 3T3 cells.

Next, several cotransfection experiments were preformed to localize CD34 promoter region elements that are involved in the nucleolin-dependent activity. For this purpose, I tested two deletion constructs pGL3/CD34^{del2}-luc and pGL3/CD34^{del3}-luc in cotransfection assays with human nucleolin expression plasmid. The promoterless pGL3Basic vector was used as a baseline control.

In NIH 3T3 cells, the wide type human CD34 promoter construct pGL3/CD34^{wt}-luc (from -666 nt. to +174 nt.) was consistently active and the relative luciferase activity of pGL3/CD34^{wt}-luc (Figure 5, column 2) was about 3.8-fold in comparison with that of the empty luciferase report vector pGL3Basic (Figure 5, column 8), whereas the activities of the deletion constructs pGL3/CD34^{del2}-luc and pGL3/CD34^{del3}-luc were decreased. The activity of pGL3/CD34^{del2}-luc (Figure 5, column 4) was only moderately decreased, and amounted 2.6-fold in comparison with that of the pGL3Basic vector (Figure 5, compare column 4 to column 8). In the cotransfection experiment, pGL3/CD34^{del2}-luc with the nucleolin expression plasmid produced only a slight increase in luciferase activity in comparison with that of the transfection using pGL3/CD34^{del2}-luc only (Figure 5, compare column 3 to column 4). However, the transfection of pGL3/CD34^{del3}-luc resulted in significant decrease in the promoter activity. The relative activity of pGL3/CD34^{del3}-luc to that of the vector pGL3Basic was about 1.7-fold (Figure 5, compare column 6 to column 8). Cotransfection of the pGL3/CD34^{del3}-luc with nucleolin expression plasmid (Figure 5, column 5) showed almost no change in luciferase activity in comparison with cotransfection of pGL3/CD34^{del3}-luc with the pCMVTag2B vector (Figure 5, column 6). These data suggest that the sequence from –367nt. to –363 nt.

of the human CD34 promoter region is required for the nucleolin-dependent activation, and this sequence is also required for the activity of the human CD34 promoter region in NIH 3T3 cells.

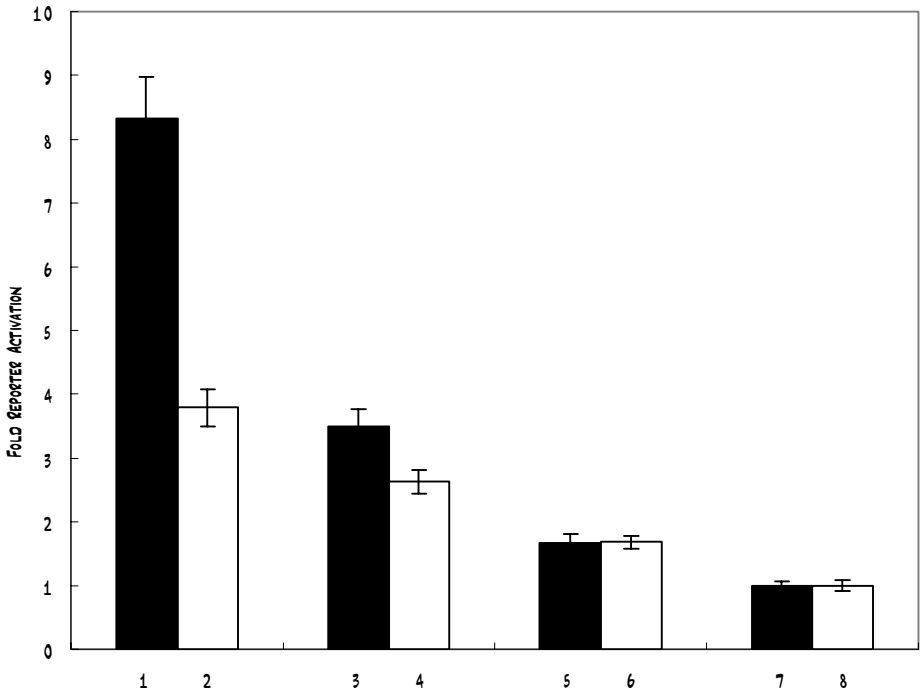

Figure 5. Analysis of the effects of nucleolin overexpression on the activity of deletion mutants of the human CD34 promoter region in NIH 3T3 cells. Cotransfection the NIH 3T3 cells using human nucleolin expression plasmid with pGL3/CD34^{wt}-luc (column 1), pGL3/CD34^{del2}-luc (column 3), or pGL3/CD34^{del3}-luc (column 5), respectively those show in Black bars (■). Cotransfection of the nucleolin expression plasmid and the pGL3Basic was used as the baseline control (column 7). Cotransfection the cells using the empty pCMVTag2B vector with the human CD34 constructs pGL3/CD34^{wt}-luc (column 2), pGL3/CD34^{del2}-luc (column 4), or pGL3/CD34^{del3}-luc (column 6), respectively that show in white bars (□). Cotransfection with

pGL3Basic vector and the empty expression vector pCMVtag2B was used as the baseline control (column 8). After 48 hours transfection, luciferase activity was measured and normalized to protein concentration. Each column represents the mean of five independent transfections. Error bars indicate the standard deviations.

Analysis of the activity of the human CD34 promoter region in stable transfected NIH 3T3 cell lines.

Transfection experiments were preformed to analyze CD34 promoter region in the stable transfected NCL-3T3 cells and CMV-3T3 cell lines. These stable transfected cell lines were used for transient transfection with pGL3/CD34^{wt}-luc and pGL3Basic, respectively. The human CD34 promoter construct pGL3/CD34^{wt}-luc was active in the CMV-3T3 cells (Figure 6, columns 2), and the activating was about 4-fold in comparison to that of the pGL3Basic vector (Figure 6, columns 4). In the NCL-3T3 cells, the luciferase activity of pGL3/CD34^{wt}-luc (Figure 6, column 1) was an averagely 8-fold in comparison to that of the pGL3Basic vector (Figure 6, column 3). These results are in line with those of the cotransfection assays shown in Figures 3 and 5.

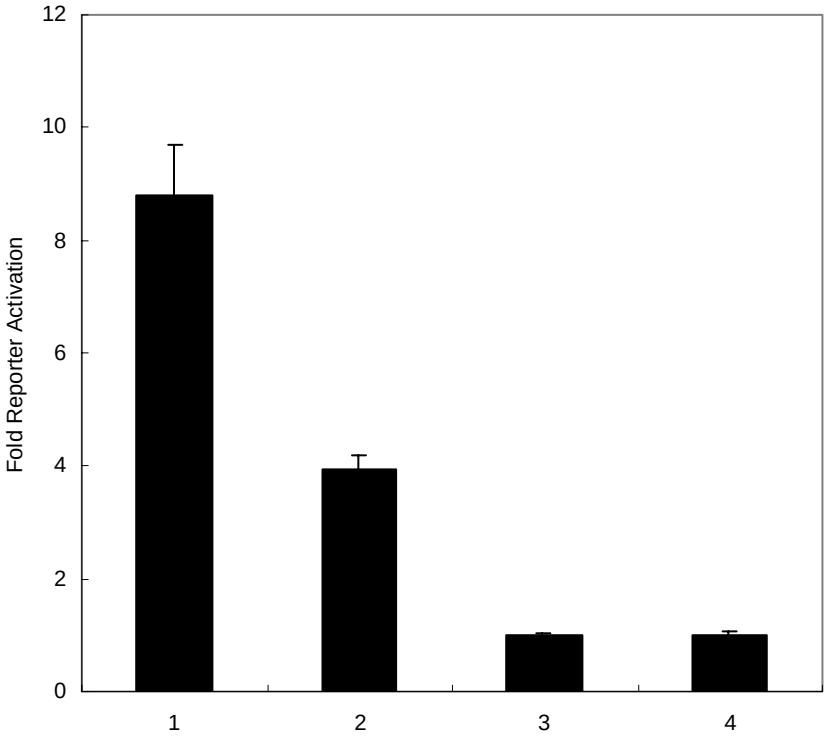

Figure 6. Analysis of the effects of nucleolin overexpression on the activity of the CD34 promoter region in stable transfected NIH 3T3 cell lines. NIH 3T3 cells were stably transfected with the human nucleolin expression plasmid (pCMV/nucleolin) or the empty expression vector (pCMVTag2B), referred to NCL-3T3 cells and CMV-3T3 cells. These two stable transfected cell lines were used for transient transfections with the plasmid pGL3/CD34^{wt}-luc and pGL3Basic, respectively. In NCL-3T3 cells, the activity of pGL3/CD34wt-luc construct (column 1) was 8.4-fold in comparison to that of pGL3Basic (column 3). In CMV-3T3 cells, the activity of pGL3/CD34wt-luc (column 2) was 3.9-fold in comparison to that of pGL3Basic (column 4). After 48 hours transient transfection, luciferase activities were measured and normalized to the protein concentration. Each column represents the mean of five independent transient transfections. Error bars indicate the standard deviations.

Analysis of the activity of deletion mutants of the CD34 promoter region in KG-1a cells.

To analyze the relationship between nucleolin and the human CD34 promoter region in human CD34 positive cell lines, the human myelogenous leukemia cell line KG-1a was transfected with pGL3/CD34^{wt}-luc, pGL3/CD34^{del2}-luc, or pGL3/CD34^{del3}-luc either with the nucleolin expression plasmid or with the vector pCMVTag2B, respectively. As shown in Figure 7, the luciferase activity of the pGL3/CD34^{wt}-luc construct (Figure 7, column 2) amounted to approximately 1.6-fold as compare with that of pGL3Basic (Figure 7, column 8). The luciferase activity measured after cotransfection with nucleolin expression plasmid and pGL3/CD34^{wt}-luc was approximately 1.8-fold as compared with the activity to pGL3/CD34^{wt}-luc (Figure 7, columns 1 and 2). The activity of pGL3/CD34^{del2}-luc construct was evidently decreased. The relative luciferase activity of pGL3/CD34^{del2}-luc (Figure 7, column 4) was about 1.2-fold as compared to that of the pGL3Basic vector (Figure 7, column 8). The activity of pGL3/CD34^{del2}-luc cotransfected with the nucleolin expression plasmid was almost not increased in comparison to that of the pGL3/CD34^{del2}-luc (Figure 7, compare column 3 to column 4). The relative luciferase activity was about 1.3-fold as compared with that of the pGL3Basic (Figure 7, compare column 3 to column 7). These data suggest that the sequence between nucleotide – 431 to – 427 of the CD34 5'flanking region participates in the nucleolin-dependent activation of the CD34 promoter. The activity of pGL3/CD34^{del3}-luc (Figure 7, column 6) construct was evidently decreased and was similar to that of the pGL3Basic vector (Figure 7, column 8). The activity of pGL3/CD34^{del3}-luc was not altered when this construct was cotransfected with nucleolin expression plasmid (Figure 7, Column 5). These data indicate that the sequence from –367nt. to –363 nt. of the human

CD34 promoter region is both important for the activity of the CD34 gene promoter region and for the nucleolin-dependent activation of the CD34 promoter region in KG-1a cells.

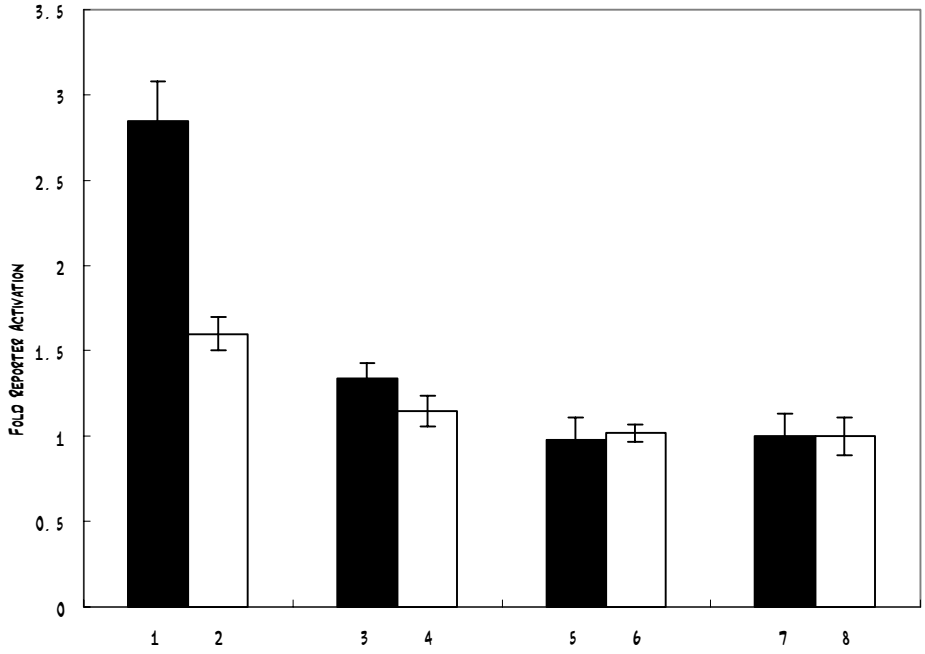

Figure 7. Analysis of the effects of the nucleolin overexpression on the activity of deletion mutants of the human CD34 promoter region in KG-1a cells. KG-1a cells were cotransfected with pGL3/CD34^{wt}-luc, pGL3/CD34^{del2}-luc, or pGL3/CD34^{del3}-luc and either with the human nucleolin expression plasmid (■) or with a control vector pCMVTag2B (□). Cotransfection the cells using human nucleolin expression plasmid with pGL3/CD34^{wt}-luc (column 1), pGL3/CD34^{del2}-luc (column 3), or pGL3/CD34^{del3}-luc (column 5), respectively. Cotransfection of the nucleolin expression plasmid with the pGL3Basic was used as the baseline control (column 7). The cells were cotransfected using the empty pCMVTag2B vector with the human CD34 constructs pGL3/CD34^{wt}-luc (column 2), pGL3/CD34^{del2}-luc (column 4), or

pGL3/CD34^{del3}-luc (column 6), respectively. Cotransfection with pGL3Basic vector and the empty expression vector pCMVtag2B was used as the baseline (column 8). After 48 hours transfection, luciferase activity was measured and normalized to protein concentration. The columns are average of transfections performed in triplicate. Error bars indicate the standard deviations.

Activity of the human CD34 promoter region is dependent on the amounts of nucleolin in KG-1a cells.

To further investigate the relationship between nucleolin and the activity of human CD34 promoter region in KG-1a cells, the activity of the pGL3/CD34^{wt}-luc construct was measured in the presence of different amounts of nucleolin expression plasmid. As shown in Figure 8, the activity of the pGL3/CD34^{wt}-luc construct was nucleolin dose-dependent (Figure 8, columns 2, 3, and 4). These data further indicate the involvement of nucleolin in activation of the human CD34 promoter region.

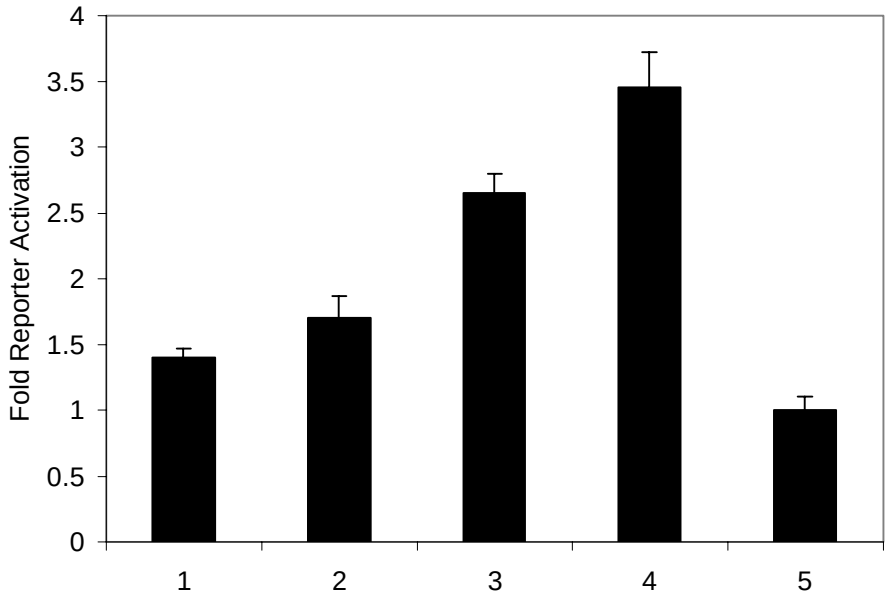

Figure 8 Activation of the human CD34 promoter region by nucleolin in KG-1a cells. KG-1a cells were cotransfected with 3 µg of pGL3/CD34^{wt}-luc and different amounts of nucleolin expression plasmid, respectively. The amounts of nucleolin expression plasmid used in the cotransfection assays were 0.1 µg (column 2), 0.3 µg (column 3), and 0.5 µg (column 4), respectively. Cotransfection of pGL3Basic vector with the empty expression vector pCMVtag2B was used as the baseline control (column 5). The cells were also cotransfected with pGL3/CD34^{wt}-luc and pCMVtag2B vector (column 1). After 48 hours transfection, Luciferase activity was measured and normalized to protein concentration. The columns are average of transfections performed in triplicate ± S.E. Error bars indicate the standard deviations.

Analysis the activity of deletion mutants of the CD34 promoter region in KG-1 cells.

The human CD34 positive cell line KG-1 was also used for the analysis of the relationship between nucleolin and the human CD34 promoter region. KG-1 cells were transfected with pGL3/CD34^{wt}-luc, pGL3/CD34^{del2}-luc, or pGL3/CD34^{del3}-luc either with the nucleolin expression plasmid or with the vector pCMVTag2B, respectively. As is shown in Figure 9, the luciferase activity of the pGL3/CD34^{wt}-luc (Figure 9, columns 2) construct amounted to approximately 1.6-fold compare with that of pGL3Basic (Figure 9, columns 8). After cotransfection of nucleolin expression plasmid, the activity of the pGL3/CD34^{wt}-luc construct was approximately 2.6-fold compared with the activity to pGL3/CD34^{wt}-luc (Figure 9, compare column 1 to column 2). The activities of pGL3/CD34del2-luc and pGL3/CD34del3-luc constructs were decreased and were similar to that of the pGL3Basic vector (Figure 9, columns 4, 6, and 8). The luciferase activities of the pGL3/CD34^{del2}-luc or pGL3/CD34^{del3}-luc cotransfection with the nucleolin expression plasmid were not increased by nucleolin (Figure 9, columns 3 and 5). These data indicate that the sequences from –367nt. to –363 nt. of the CD34 promoter region deleted in the pGL3/CD34^{del3}-luc construct and the sequences from nucleotide –431 to – 427 of the CD34 promoter region are important for the activity of the CD34 gene and are involved in the nucleolin-dependent activation of the CD34 promoter region in KG-1 cells.

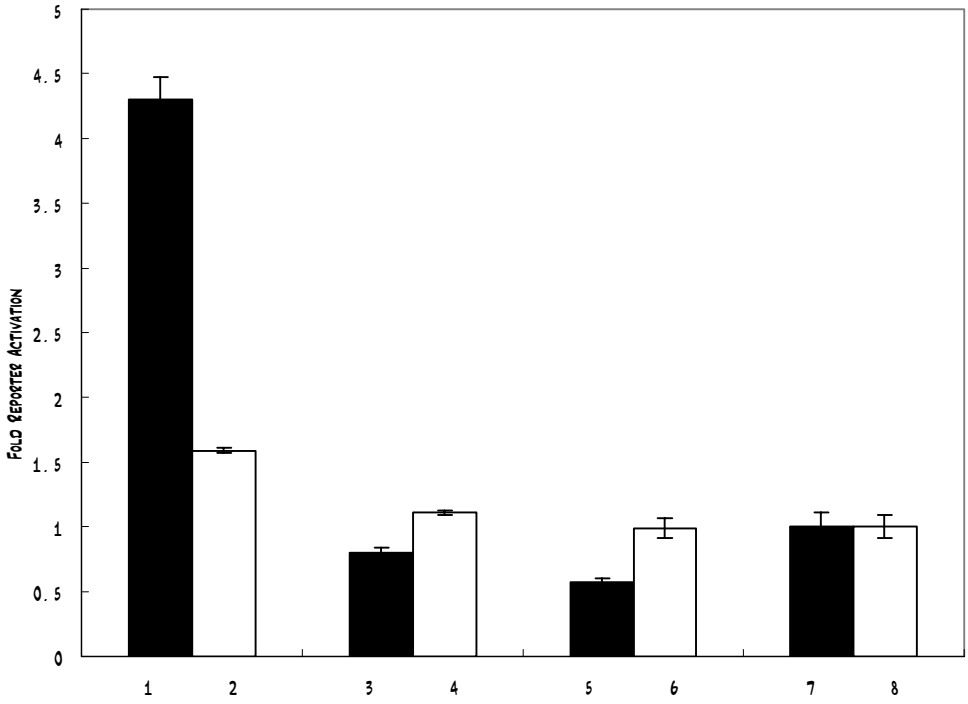

Figure 9. Analysis of the effects of nucleolin overexpression on the activity of deletion mutants of the human CD34 promoter region in KG-1 cells. KG-1 cells were cotransfected with human CD34 promoter constructs either with the human nucleolin expression plasmid (■) or with the empty pCMVTag2B vector (□). Cotransfection the cells using human nucleolin expression plasmid with pGL3/CD34^{wt}-luc (column 1), pGL3/CD34^{del2}-luc (column 3), or pGL3/CD34^{del3}-luc (column 5), respectively. Cotransfection the cells with nucleolin expression plasmid and the pGL3Basic was used as the baseline control (column 7). Cotransfection the cells using the empty pCMVTag2B vector with the human CD34 constructs pGL3/CD34^{wt}-luc (column 2), pGL3/CD34^{del2}-luc (column 4), or pGL3/CD34^{del3}-luc (column 6), respectively. Cotransfection using pGL3Basic vector and the empty expression vector pCMVtag2B was used as the baseline (column 8). The luciferase activities were normalized to protein concentration and

are shown in comparison with the activity of the promoterless pGL3Basic vector. (bars ± SE.) The values are average of transfections of preformed in triplicate. Error bars indicate the standard deviation.

Discussion

It was the aim of this study to evaluate an involvement of nucleolin in regulation of the human CD34 gene promoter region. In this investigation, I have shown that the human nucleolin can activate the human CD34 promoter region in the nonhematopoietic CD34 positive murine fibroblast NIH 3T3 (Figure 3, 4, and 5) and in the hematopoietic CD34 positive human cell lines KG-1 (Figure 9) and KG-1a (Figure 7 and 8). These data indicated that the activity of the human CD34 promoter region depends on the presence of the fragment between nucleotide –470 and –344 (Figure 3). There are several elements for transcription regulation proteins that could be responsible for the observed change in the CD34 promoter region activity. Three putative Myb motifs (AAGTTA) and one Ets-like binding motif (AGGAA) have been identified between –470 nt. and –344 nt. within the human CD34 promoter region (Burn, Satterthwaite et al. 1992; He, Antao et al. 1992). Both Ets and c-Myb are involved in activation of CD34 gene expression in the hematopoietic cell lines (Melotti and Calabretta 1994; Melotti, Ku et al. 1994). One of the Myb binding motifs, which is located within the 127 nt. fragment (between –470 nt. and –344 nt.) has been demonstrated to have important function in transcription regulation of CD34 gene expression (Melotti and Calabretta 1994; Melotti, Ku et al. 1994). Deletion of the Myb transcription factor binding motif within the 127 bp fragment in the human CD34 promoter region would therefore in part explain the decrease of the CD34 promoter activity (Figure 1B and 3), further investigations are required. In this study, experiments have been preformed to identify whether human CD34 gene promoter region can be activated by nucleolin, by means of cotransfection assays.

An importance of two elements within the human CD34 promoter region for the nucleolin-dependent activation can be suggested. Deletion of the sequence motif between –367 nt. and –363 nt. of the human CD34 promoter region abolishes both the human CD34 promoter activity and the nucleolin-dependent activation of the human CD34 promoter region. These data indicate that the sequence (between – 367nt. and –363 nt.) or a neighbor CD34 promoter region sequence (between – 431nt. and –427 nt.) is important for the nucleolin-dependent activation of the human CD34 promoter region. It was shown previously that the region from –368 nt. to –354 nt. of the human CD34 promoter region was protected from DNase I digestion (Perrotti, Bellon et al. 1996), and a corresponding nuclear multiprotein complex, NC-3A was described to positively regulate the human CD34 promoter region (Perrotti, Bellon et al. 1996). Although the nature of the multiprotein complex, NC-3A is not known, it was shown that the multiprotein complex contains at least three distinct proteins binding with this region and positive regulation the CD34 gene expression (Perrotti, Bellon et al. 1996). Further experiments are required to establish the relationship between the data presented here and those of Perrotti and his colleagues. The interaction between nucleolin and transcription factor c-Myb has been reported previously. The c-myb gene is highly expressed in immature hematopoietic cells and its expression is down-regulated during differentiation (Gonda and Metcalf 1984; Duprey and Boettiger 1985; Ramsay, Ikeda et al. 1986; Sitzmann, Noben-Trauth et al. 1995). It has also been shown that c-Myb regulate CD34 gene expression by binding to Myb binding site in the CD34 promoter region (Melotti and Calabretta 1994). Nucleolin affects Myb transcriptional activity by binding to the DNA binding domain of the A-Myb and C-Myb transcription factors (Ying, Proost et al. 2000). Thus, it is possible that nucleolin may also be involved in regulation of the CD34 promoter region via an interaction with the c-Myb transcription factor for a co-operative

action. Further work will be required to elucidate this possibility. In this study, we find that yet another sequence motif with sequence CTTGC (between – 431 nt. and –427 nt.) of the human CD34 promoter region is probably also involved in nucleolin-dependent activation. This element is present adjacent to a putative Ets binding site in the CD34 promoter region (Burn, Satterthwaite et al. 1992; He, Antao et al. 1992). Therefore, a co-operative action of nucleolin and the Ets transcriptional factor might be expected. Further experiments are needed to evaluate this possibility.

To analyze a role of nucleolin in the modulation of the amounts of CD34 glycoprotein at the cell surface in a CD34 positive cell line, a NCL-KG1 cell line, stable transfection with a nucleolin expression plasmid was performed. The flow cytometry analysis results show that constitutive overexpression of nucleolin in NCL-KG1 revealed the presence of increased amounts of the CD34 glycoprotein at the cell surface (data not shown Dr. Grinstein personal communication). These data suggest a role of nucleolin in regulation of CD34 gene expression. Nucleolin is an abundant protein in growing eukaryotic cells (Lapeyre, Bourbon et al. 1987) and it is highly expressed in CD34 negative cell lines, as exemplified by HeLa, SiHa cell lines (Grinstein, Wernet et al. 2002) and certain other cells such as mature neuronal cells contain nucleolin (Kibbey, Johnson et al. 1995), but they are CD34 negative cells. This implies that other mechanisms are responsible for the tissue-specificity of the CD34 gene expression.

Nucleolin is thought to be a multifunctional phosphoprotein. Several functions have been identified for nucleolin: The central region of nucleolin is the RNA binding region, which contains four RNA binding domains that responsible for RNA-binding specificity (Bourbon, Lapeyre et al. 1988; Srivastava, Fleming et al. 1989). The acidic N-terminus has

been implicated in interaction with DNA and histone H1 (Erard, Belenguer et al. 1988; Hanakahi, Dempsey et al. 1997). The synergistic effect of the phosphorylated nucleolin and histone H1 on chromatin condensation has been described (Kharrat, Derancourt et al. 1991). Histone H1 phosphorylation has been shown to activate or repress specific genes (Dou and Gorovsky 2000). Thus, these data have led to the proposal that nucleolin and its phosphorylation may be involved in various events with other factors to gain access to DNA in chromatin. In addition, it has been demonstrated that nucleolin is one of the component of LR1 (Hanakahi, Dempsey et al. 1997). LR1 is a sequence-specific DNA binding protein with a heterodimer composition in B cells (Williams and Maizels 1991; Williams, Hanakahi et al. 1993). LR1 play roles in positive regulation the c-Myc transcription in B-cell lymphomas (Brys and Maizels 1994) and also appears to function in class switch recombination in B cells (Hanakahi, Dempsey et al. 1997; Hanakahi and Maizels 2000). Another component of LR1 is a 45-kDa polypeptide which has been identified was the hnRNP D in pre-B and B cells (Williams, Hanakahi et al. 1993; Hanakahi, Dempsey et al. 1997; Dempsey, Hanakahi et al. 1998). In particular, the LR1 DNA binding activity dependents on its phosphorylation and post-translational modification by N-acetylglucosamine (Williams and Maizels 1991; Williams, Hanakahi et al. 1993), which may important in regulating LR1 activity. The structure analysis shows that both hnRNP D and nucleolin contain RNA binding domains (RBDs) and Arg-Gly-Gly (RGG) motifs. Although these motifs are not commonly associated with sequence-specific recognition of DNA, it is likely that nucleolin and hnRNPs can combine to produce a sequence specific DNA-binding activity (Dempsey, Hanakahi et al. 1998). It is likely that nucleolin combine to some other protein components in regulating genes transcription.

In summary, this thesis has shown that the human nucleolin is involved in positive regulation the human CD34 gene promoter region in CD34 positive cells and identified two DNA motifs that are necessary for the human CD34 promoter region activity. Nucleolin is likely to be involved in the transcriptional regulation of the human CD34 gene in CD34 positive cell lines. Analysis of nucleolin interactions with the CD34 gene regulation region, as well as with other proteins that modulate transcriptional activity of the CD34 gene may be required for further interpretation of these model findings.

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Papers Published in Chinese before 2002:

Yihua Du, Shiro Manabe, and Norihiro Nakamura et al: Identification of Novel Mouse GUE cDNA Transcribed from Upstream Region of GATA-6 Gene. Annual Report of International Center Cooperative Research in Biotechnology Japan (2002)

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