Vitamin D₃ promotes myeloid leukemic cell lines to differentiate terminally into monocytes/macrophages. It has been reported that overexpression of the cdk inhibitor p27Kip₁ results in the differentiation of the monolymphocytic U937 cell line and that this gene is the target of vitamin D₃. To identify the sequences required for the positive regulation of p27Kip₁ transcription by vitamin D₃, a 3.6-kilobase 5'-flanking region of the human p27Kip₁ gene was examined by transiently transfecting luciferase reporter constructs into U937 cells. The transcriptional activity of this construct was activated by vitamin D₃. Deletion and mutational analysis revealed that both a GGCGG sequence (−545/−539) and a CCAAT sequence (−525/−520) were necessary to induce p27Kip₁ gene expression. Importantly, the region containing both of these elements conferred positive responsiveness to vitamin D₃ to a heterologous promoter. Gel shift assays showed that Sp1 binds to the GGCGG sequence and that NF-Y binds to the CCAAT promoter. Gel shift assays showed that Sp1 binds to the enhancer-binding protein.

CTF, CCAAT box binding transcriptional factor; C/EBP, CCAAT/ min D response element; bp, base pair; PCR, polymerase chain reaction.

Vitamin D₃ induces cell differentiation. For instance, monocyte differentiation is mediated by vitamin D₃ without requiring binding to VDR (7), and keratinocyte differentiation-related genes are stimulated by vitamin D₃ without the presence of VDR (8). Clarification of the regulatory mechanisms of p27Kip₁ transcription is crucial for the understanding of the molecular mechanisms of vitamin D₃ action and for the understanding of the early processes during monocyte/macrophage differentiation. Although the post-transcriptional regulation of p27Kip₁ has been studied intensively (9–11), very little is known about the transcriptional regulation of the p27Kip₁ gene.

This study, we have analyzed the p27Kip₁ promoter in order to identify elements required for vitamin D₃-induced up-regulation of transcription, which might reveal the early and novel mechanism of differentiation induction by vitamin D₃.

**MATERIALS AND METHODS**

**Construction of the Luciferase Reporter Plasmid**—The promoter region of the human p27Kip₁ gene (p27PF) or enzyme-generated 5'-deletion sequences (p27A, p27B, p27C, p27D) were subcloned into the pGL2 Basic vector (Promega) as described previously (4). The plasmids with point mutations in p27PF (p27mSp1-1, p27mSp1-2, and p27mCTF) were generated by in vitro mutagenesis as described previously (12). Furthermore, we generated constructs containing four tandem copies of the specific sequence of the p27Kip₁ promoter fused to a minimal promoter. A double-stranded 52-bp DNA fragment containing a 44-bp sequence corresponding to the −555/−512 region of the human p27Kip₁ promoter and linker sites (indicated by lowercase letters below) at both ends was generated from two oligonucleotides. The top strand (−555/−512) and the bottom strand (−555/−512) were annealed, ligated, blunted, and subcloned into the Smal site of the SV40 early promoter in the PicaGene Promoter Vector 2 (Nippon Gene, Tokyo, Japan) in a forward or reverse orientation to generate PGFPV2[−555/−512wild] and PGFPV2[−555/−512wild] respectively. Similarly, three types of double-stranded mutated oligonucleotides of the 44-bp sequence were also inserted the same vector to generate the mutated constructs PGFPV2[−555/−512mSp1-1], PGFPV2[−555/−512mSp1-2], and PGFPV2[−555/−512mCTF] (see Table I).

**Cell Culture and Differentiation Induction**—The human myelomonocytic U937 cell line and that this gene is the target of vitamin D₃. To identify the sequences required for the positive regulation of p27Kip₁ transcription by vitamin D₃, a 3.6-kilobase 5'-flanking region of the human p27Kip₁ gene was examined by transiently transfecting luciferase reporter constructs into U937 cells. The transcriptional activity of this construct was activated by vitamin D₃. Deletion and mutational analysis revealed that both a GGCGG sequence (−545/−539) and a CCAAT sequence (−525/−520) were necessary to induce p27Kip₁ gene expression. Importantly, the region containing both of these elements conferred positive responsiveness to vitamin D₃ to a heterologous promoter. Gel shift assays showed that Sp1 binds to the GGCGG sequence and that NF-Y binds to the CCAAT promoter. Gel shift assays showed that Sp1 binds to the enhancer-binding protein.

**CTF, CCAAT box binding transcriptional factor; C/EBP, CCAAT/ min D response element; bp, base pair; PCR, polymerase chain reaction.**
Regulation of p27Kip1 Transcription by Vitamin D₃

**TABLE I**

Oligonucleotides used in this study

| Oligomer | Sequence | Ref. |
|----------|----------|------|
| −555/−512wild | 5'-aggAAAGGCAGCGGCGGCGTTGCTGTCG-3' | This study |
| Sp1-1 | 5'-TAAGCTAGGATGGGCGAGC-3' | 41 |
| Sp1-2 | 5'-ATTCGATCGGGATGGGGCGAGC-3' | 41 |
| −555/−512mSp1-1 | 5'-aggAAAGGCAGCGGCGGCGTTGCTGTCG-3' | This study |
| −555/−512mSp1-2 | 5'-TAAGCTAGGATGGGCGAGC-3' | This study |
| −555/−512mCTF | 5'-ATTCGATCGGGATGGGGCGAGC-3' | This study |
| −534/−512wild | 5'-ATTCCGATCGGGCGGCGGAGC-3' | This study |
| −534/−512mCTF | 5'-ATTCCGATCGGGCGGCGGAGC-3' | This study |
| Sp1wild | 5'-ATTCCGATCGGGCGGCGGAGC-3' | 19 |
| Sp1mt | 5'-ATTCCGATCGGGCGGCGGAGC-3' | 19 |
| NF-Ywild | 5'-ATTCCGATCGGGCGGCGGAGC-3' | 22 |
| NF-1wild | 5'-ATTCCGATCGGGCGGCGGAGC-3' | 22 |
| NF-1mt | 5'-ATTCCGATCGGGCGGCGGAGC-3' | 22 |

**RESULTS**

**Vitamin D₃-responsive Elements in the Human p27Kip1 Promoter**—It has been reported that p27Kip1 and p21Cip1 are transcriptionally induced by vitamin D₃ (2). We also observed that p27Kip1 mRNA was induced after treatment of U937 cells with vitamin D₃, and it peaked between 24 h and 48 h (approximately 4-fold compared with 0 h), whereas p21Cip1 mRNA induction was rapid (data not shown). This suggests that vitamin D₃ regulates transcription of p27Kip1 in a VDR/VDRE-independent manner unlike the case of p21Cip1 transcription. To investigate the regulatory mechanisms behind p27Kip1 gene expression, we first investigated the effect of vitamin D₃ on the transcriptional activity of the promoter of the p27Kip1 gene. The effect of vitamin D₃ on the wild type p27Kip1 promoter-lucif-
**Fig. 1. Differential effects of vitamin D₃ on the human p27Kip₁ promoter activities with a series of 5'-deletions.** 18 µg of each constructed plasmid was transiently transfected into U937 cells with 2 µg of pRL-TK, and luciferase activities were analyzed after a 40-h treatment of 10⁻¹⁰ M vitamin D₃. Relative luciferase activities are shown as percentages of that of p27PF in the absence of vitamin D₃. Data are shown as means (bars, standard deviation) (n = 3). *, p < 0.01; **, p < 0.05.

A 44-bp Regulatory Sequence between −555 and −512 Which Contains the Sp1-1 Site and the CCAAT Box Confers Response to Vitamin D₃ to a Heterologous Promoter—To examine vitamin D₃ regulation via the Sp1-1 site and the CCAAT box that are located near each other downstream from −555, four tandem copies of the sequences corresponding to −555/−512 with or without mutations of the Sp1-1 site, the Sp1-2 site, or the CCAAT box were inserted upstream of the SV40 early promoter in the PicaGene Promoter Vector 2 in a normal (PGPV2[−555/−512wild]₄), PGPV2[−555/−512mSp1-1]₄, PGPV2[−555/−512mSp1-2]₄, PGPV2[−555/−512mSp1-2]₄, and PGPV2[−555/−512 mCTF]₄ or a reverse orientation (PGPV2[R−555/−512wild]₄) (Fig. 3). As shown in Fig. 3, four copies of the 44-bp fragment corresponding to between −555 and −512 of the p27Kip₁ promoter conferred significant response to vitamin D₃ to the SV40 early promoter in an orientation-dependent manner following transient transfection of U937 cells, although one copy of the same fragment did not respond to vitamin D₃ (data not shown). Mutations introduced in either the Sp1-1 or the CCAAT box (PGPV2[−555/−512mSp1-1]₄, PGPV2[−555/−512mCTF]₄, respectively) abolished any stimulatory effect by vitamin D₃, whereas luciferase activity of a construct containing mutations in the Sp1-2 site (PGPV2[−555/−512mSp1-2]₄) was activated by vitamin D₃ in a manner similar to that of p27PF and PGPV2[−555/−512wild]₄. We concluded that the region between −555 and −512 relative to the transcription start site (−118 and −75 relative to the transcription start site²) was sufficient for vitamin D₃-induced transcription of the p27Kip₁ gene, although we could not exclude the possibility that other sequences in the p27Kip₁ promoter were also required. Furthermore, both the Sp1-1 site and the CCAAT box in this region were vitamin D₃-responsive elements and were required for vitamin D₃-induced transcription of the p27Kip₁ gene. 

Identification of Nuclear Proteins Interacting with the Vita-

² J. Kamiyama, unpublished data.
min D3-responsive Sequence—To identify the nuclear factors binding to the vitamin D3-responsive sequence, a set of oligonucleotides spanning −555 to −512 was used as a probe for gel shift assays (−555/−512wild, see Table I). Nuclear extracts were prepared from U937 cells treated with vitamin D3 for 36 h. As shown in Fig. 4A, the oligonucleotides −555/−512wild yielded a single major retarded band (lane 1), which was competed away by an excess of unlabeled oligonucleotide (lanes 2 and 3). To localize the sequence that binds to nuclear factor(s), a series of oligonucleotides that carried point mutations in the Sp1-1 site, the Sp1-2, or the CCAAT box (Sp1wild, Sp1mt, and C/EBP-α wild), and oligonucleotides spanning −534 to −512 were used as competitors. As shown in lanes 4–17, the retarded band was not competed out by the addition of −555/−512mSp1-1, Sp1mt, NF-Ywild, or −534/−512wild, indicating that Sp1 family protein(s) bind to the Sp1-1 site. To elucidate whether the retarded band represents the binding of Sp1 or Sp3, gel shift assays were performed with nuclear extracts that were preincubated with anti-Sp1 or -Sp3 antibody for band supershift experiments (18). As shown in Fig. 4B, in the presence of anti-Sp1 antibody but not anti-Sp3 antibody, the complex was supershifted. We concluded that Sp1 binds to the Sp1-1 site of the vitamin D3 regulatory region of the p27Kip1 promoter in U937 cells.

We also analyzed the sequence between −534 and −512 which carries the CCAAT box but not the Sp1-1 and Sp1-2 sites. A set of oligonucleotides spanning this region was used as a probe for gel shift assays. As shown in Fig. 5A, we observed a single major retarded band, which was competed away by excess unlabeled wild type oligonucleotides (−534/−512wild) but not those carrying a mutation in the CCAAT box (−534/−512mCTF) (lanes 1–5). This indicated that nuclear factor(s) bind to the CCAAT box of the p27Kip1 promoter. So far, it has been reported that several different transcriptional factors including NF-Y, C/EBP, CAT-binding protein, and NF-I are capable of binding to CCAAT sequences (19–22). To elucidate which transcription factor binds to the CCAAT box in the p27Kip1 promoter, we performed competition experiments using unlabeled oligonucleotides carrying the CCAAT sequence that had been reported to bind to NF-Y, C/EBP-α, or NF-I with a high affinity (NF-Ywild, C/EBP-owlid, and NF-Iwild, respectively). As shown in Fig. 5A, the retarded band was competed by the addition of NF-Ywild but not NF-Iwild or C/EBP-owlid. This suggested that NF-Y binds to this CTF site. To confirm this result, we performed supershift assays using anti-NF-Y antibodies. NF-Y is composed of three subunits, NF-YA, NF-YB, and NF-YC. We used anti-NF-YA,
-YB, and -YC antibodies (15, 23) and anti-C/EBP-α antibody for supershift experiments. As shown in Fig. 5B, in the presence of anti-NF-YA, -YB, or -YC antibodies, the complex was super-shifted. The addition of anti-C/EBP-α did not affect the complex. These results demonstrated that trimeric NF-Y binds to the CCAAT box of the regulatory region of the p27Kip1 promoter, although we could not observe NF-Y binding to 555–512wild probe. To verify that NF-Y could bind not only to the CCAAT box in the 555–512wild sequence but also to the site in the 534–512wild sequence, we again used oligonucleotides spanning 555 to 512, with or without mutations in the Sp1-1, Sp1-2, or CTF site as competitors. For the same purpose, we investigated the effects of the addition of anti-NF-YA, -YB, or -YC antibodies on the formation of the complexes of 555–512wild probe and nuclear proteins by gel shift assays. The addition of anti-NF-YA, -YB, or -YC antibody did not affect the complexes of 555–512wild and nuclear proteins (data not shown). However, as shown in Fig. 5A, NF-Y binding to the probe 534/512wild was competed by 555/512wild, 555/512mSp1-1, and 555/512mSp1-2, although these oligonucleotides were less effective than 534/512wild, while NF-Y binding to 534/512wild was not affected by 555/512mCTF. These results indicated that NF-Y binds to the CCAAT box in 555/512wild as well as the site in 534/512wild. Therefore, we concluded that Sp1 and NF-Y bind to the Sp1-1 site and the CCAAT box, respectively, the regulatory elements that are required for vitamin D3-induced transcription of the p27Kip1 gene.

To confirm further the involvement of NF-Y in the vitamin D3-induced transcription of p27Kip1, we cotransfected p27PF or p27mCTF with a dominant negative NF-YA mutant expression plasmid (pNF-YA29) (24). As shown in Fig. 6, pNF-YA29 suppressed the vitamin D3-induced luciferase activities from p27PF but not p27mCTF in a dose-dependent manner. This result demonstrates directly that NF-Y mediates the up-regulation of p27Kip1 transcription by vitamin D3 via the CTF site of its promoter.

Analysis of Sp1 and NF-Y Subunits after Vitamin D3 Treatment—The results described above suggested strongly that Sp1 and NF-Y are the essential regulators in vitamin D3-induced transcription of the p27Kip1 gene. To investigate the mechanism how Sp1 and NF-Y regulate transcription of the p27Kip1 gene, we examined whether binding of Sp1 and NF-Y to the p27Kip1 promoter was altered following vitamin D3 treatment,
using gel shift assays. Nuclear extracts were prepared from U937 cells treated with either vitamin D₃ or vehicle alone for 36 h. As a probe, -555/-512wild oligonucleotides or -534/-512wild oligonucleotides were used to detect Sp1 and NF-Y, respectively. As shown in Fig. 7, we observed that Sp1 and NF-Y binding activities increased significantly after vitamin D₃ treatment. Because the DNA binding activity of an unrelated transcription factor, NF-I, was not changed by the treatment of vitamin D₃, we concluded that the treatment of vitamin D₃ specifically stimulates the binding of Sp1 and NF-Y to the p27Kip1 promoter. To analyze the protein levels of Sp1 and NF-Y subunits, whole cell extracts were prepared from U937 cells treated with vitamin D₃ or vehicle alone at different time points. The amounts of these proteins were analyzed by Western blotting. As shown in Fig. 8, the level of Sp1 increased slightly from 12 h to 18 h after the vitamin D₃ treatment, and after 36 h the level of Sp1 was reduced and barely detectable. The increase in the level of Sp1 occurred prior to the increase of p27Kip1 mRNA following the vitamin D₃ treatment (data not shown). Interestingly, the level of Sp1 protein showed little difference between vitamin D₃ and vehicle-treated cells after 36 h, when we could observe a significant increase in the binding of Sp1 to the Sp1-1 site of the human p27Kip1 promoter (Fig. 7). These results indicate that post-translational regulation of Sp1 such as phosphorylation (25) and glycosylation (26) could contribute to up-regulation of p27Kip1 transcription by vitamin D₃ in combination with slight induction of Sp1 protein. On the other hand, as reported previously, we also observed two bands for NF-YA (36 kDa and 39 kDa) which are thought to result from differential splicing (15, 16, 27) (Fig. 8). To our surprise, the level of the 39-kDa form of NF-YA decreased from 12 h to 48 h after the vitamin D₃ treatment. To confirm that this change resulted from the decrease of long form NF-YA mRNA, we performed reverse transcriptase PCR analysis of NF-YA mRNA using primers F1 and R that were located in the regions that are conserved in the two isoforms. As shown in
Fig. 6. A dominant-negative NF-YA expression plasmid (pNF-YA29) suppresses the vitamin D₃ responsiveness of the p27 promoter. 9 μg of p27PF or p27mCTF was cotransfected into U937 cells with various amounts of expression plasmid for the dominant negative NF-YA (pNF-YA29) with 2 μg of pRL-TK. Luciferase activities were analyzed after a 40-h treatment with 10⁻⁷ x vitamin D₃ compared with p27PF without vitamin D₃ treatment. Data are shown as means (bars, standard deviation) (n = 3). *, p < 0.01.

Fig. 7. Change of Sp1 and NF-Y binding activities to the p27Kip1 promoter after vitamin D₃ treatment. Nuclear extracts were prepared from either vitamin D₃-treated or vehicle (ethanol)-treated U937 cells for 36 h. 2 μg of each nuclear extract was incubated with the −555/−512wild probe (lanes 1–3), the −534/−512wild probe (lanes 4–6), or the NF-Iwild probe (lanes 7–12) to detect Sp1, NF-Y, and NF-I, respectively.

In this study, we analyzed the vitamin D₃-induced transcription of p27Kip1 as a model to understand a possible novel pathway of vitamin D₃ action which does not directly involve VDR/VDRE and the mechanism of U937 differentiation. From transient transfection studies, we conclude that Sp1 and NF-Y mediate the vitamin D₃-induced transcription via elements that are closely located adjacent to each other in the promoter region of p27Kip1. Importantly, the 44-bp element in the p27Kip1 promoter that carries Sp1 and NF-Y binding sites is sufficient for the response to vitamin D₃, and both elements are required for this response. We believe that this is the first report that explains the molecular mechanism of vitamin D₃-induced transcription that does not directly require VDR, although vitamin D₃-dependent transcriptional repression that does not require VDR has been reported previously (28, 29).

The next question to be solved was how vitamin D₃ activated the p27Kip1 gene expression via Sp1 and NF-Y. Binding activities of Sp1 and NF-Y to each element in the p27Kip1 promoter were stimulated significantly after vitamin D₃ treatment. Furthermore, Western blotting analysis showed that Sp1 increased slightly, and one subunit of NF-Y changed to a low molecular weight form prior to the accumulation of p27Kip1 mRNA. These findings raise the hypothesis that post-translational modification of Sp1 and differential splicing of NF-YA induced by vitamin D₃ lead to the activation of p27Kip1 transcription through enhanced binding of these factors to regulatory elements in the p27Kip1 promoter. It has been shown that Sp1 can mediate responses to several inducers of myeloid differentiation, and several myeloid promoters are dependent on a functional Sp1 site (30–32). NF-Y also can mediate responses to several inducers of myeloid differentiation and macrophage maturation (15, 33, 34). However, little is known about the mechanism of how NF-Y mediates these responses. We found that the form of NF-YA changes after differentiation induced by vitamin D₃ and differential splicing of NF-YA induced by vitamin D₃ lead to the activation of p27Kip1 transcription through enhanced binding of these factors to regulatory elements in the p27Kip1 promoter. It has been shown that Sp1 can mediate responses to several inducers of myeloid differentiation, and several myeloid promoters are dependent on a functional Sp1 site (30–32). NF-Y also can mediate responses to several inducers of myeloid differentiation and macrophage maturation (15, 33, 34). However, little is known about the mechanism of how NF-Y mediates these responses. We found that the form of NF-YA changes after differentiation induced by vitamin D₃ and differential splicing of NF-YA induced by vitamin D₃ lead to the activation of p27Kip1 transcription through enhanced binding of these factors to regulatory elements in the p27Kip1 promoter. It has been shown that Sp1 can mediate responses to several inducers of myeloid differentiation, and several myeloid promoters are dependent on a functional Sp1 site (30–32).

The possible mechanism is the involvement of histone acetyltransferases, known as transcriptional cofactors including
p300, GCN5, and P/CAF (35). Recently it was shown that NF-Y interacts with p300 in vivo, and NF-Y establishes a pre-set promoter architecture that can facilitate transcription within chromatin by recruiting p300 protein (36). Similarly, NF-Y has been shown to be associated with GCN5 and P/CAF in vitro, and GCN5 activates NF-Y-dependent transcription in vitro (37). We reported previously that histone deacetylase inhibitors activate the p21Cip1 gene promoter through Sp1 sites that can interact with Sp1 and Sp3 (18, 38). Thus, it is possible that NF-Y activates p27Kip1 transcription through modification of Sp1 sites by recruiting histone acetyltransferases.

In this study, we found a novel pathway to mediate vitamin D₃ action which does not directly involve VDR. We then searched for the genes that carry Sp1 and CCAAT sequence in their promoter regions. We found that both elements exist in the promoter region of the VDR gene. This suggests that the regulatory mechanism we found might be more generally applicable to other genes that are regulated by vitamin D₃. Reverse transcriptase PCR was carried out using different combinations of PCR primers. Top panel, primers F1 and R; middle panel, primers F2 and R; bottom panel, primers for glyceraldehyde-3-P-dehydrogenase.

FIG. 8. Change of Sp1 and NF-Y subunits after vitamin D₃ treatment. Panel A, whole cell extracts were prepared from U937 cells at the indicated time points after the treatment of vitamin D₃. Extracts were subjected to 12% SDS-polyacrylamide gel electrophoresis, followed by Western blotting with anti-Sp1 or with anti-NF-YA, -YB, -YC antibodies. Panel B, total RNAs were prepared from U937 cells at the indicated time after the treatment of vitamin D₃. Reverse transcriptase PCR was carried out using different combinations of PCR primers. Top panel, primers F1 and R; middle panel, primers F2 and R; bottom panel, primers for glyceraldehyde-3-P-dehydrogenase.
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