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A Carboxy-Terminally Truncated Form of the Vpr Protein of Human Immunodeficiency Virus Type 1 Retards Cell Proliferation Independently of G_2 Arrest of the Cell Cycle

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Vpr, one of the accessory gene products of HIV-1, is a 96-residue protein with several functions. It is involved in import of the HIV-1 preintegration complex into the nucleus of nondividing cells, in cellular differentiation, inducing cell cycle arrest at the G_2/M phase, in immune suppression, and in enhancement of replication of the virus. We found recently that Vpr interferes with the proliferation of mouse NIH3T3 fibroblasts but fails to arrest these cells in the G_2 phase. Thus, it seems possible that Vpr might retard cell proliferation via a novel pathway that is distinct from G_2 arrest. To elucidate the mechanism by which Vpr induces the retardation of cell growth, we developed a panel of expression vectors that encoded Vpr molecules with deletions of specific putative domains, namely, the first α-helical domain, the second α-helical domain, a leucine zipper-like domain, and an arginine-rich carboxy-terminal domain. These vectors were introduced into HeLa cells since expression of Vpr can induce G_2 arrest in such cells. A carboxy-terminally truncated form of Vpr, C81, which failed to induce G_2 arrest, led to the G_1 arrest and retained the ability to prevent cell proliferation. All the other mutant proteins had completely lost the capacity to induce G_2 arrest and to suppress growth. Substitutions of Ile/Leu for Pro at positions 60, 67, 74, and 81 within the leucine zipper-like domain of Vpr or of C81 revealed that Ile60, Leu67, and Ile74 play an important role in the C81-induced suppression of growth, while Ile74 and Ile81 were found to be indispensable for Vpr-induced G_2 arrest. Collectively, our results strongly suggest that Vpr can retard cell proliferation independently of G_2 arrest of the cell cycle.

INTRODUCTION

The genome of human immunodeficiency virus type 1 (HIV-1) contains not only structural genes, such as *gag, pol,* and *env,* but also the accessory genes *vif, vpr, vpu,* and *nef.* One such gene, *vpr,* encodes a 15-kDa nuclear protein of 96 amino acids that is incorporated into virions through interaction with the p6 C-terminal product of Gag (Cohen *et al.,* 1990; Kondo and Gottlinger, 1996; Kondo *et al.,* 1995; Lu *et al.,* 1993). Vpr and matrix antigen (MA) act directly to promote the nuclear import of the HIV-1 preintegration complex via the karyopherin pathway (Gallay *et al.,* 1996; Popov *et al.,* 1998), thereby allowing replication in nonproliferation targets, such as terminally differentiated macrophages (Heinzinger *et al.,* 1994; Vodicka *et al.,* 1998). Vpr arrests the cell cycle at the G_2/M phase by preventing the activation of the $p34^{cdc2}$-cyclin B complex, and this inhibition appears to result from increased phosphorylation of $p34^{cdc2}$ at specific sites (Bartz *et al.,* 1996; He *et al.,* 1995; Jowett *et al.,* 1995; Re *et al.,* 1995; Rogel *et al.,* 1995). The capacity for Vpr-mediated arrest of the cell cycle is conserved among strongly divergent simian immunodeficiency viruses (SIV) (Planelles *et al.,* 1996), suggesting an important role for Vpr in the life cycle of such viruses. Indeed, the expression of the viral genome is maximal during the G_2 phase of the cell cycle, and Vpr increases the production of virus by delaying cells at the point of the cell cycle where the long terminal repeat (LTR) is most active (Felzien *et al.,* 1998; Goh *et al.,* 1998). In addition, Vpr causes the terminal differentiation of certain types of cell (Levy *et al.,* 1993) and the induction of apoptosis after cell cycle arrest (Stewart *et al.,* 1997). Furthermore, there is evidence that Vpr seems to be able to regulate apoptosis both positively and negatively (Ayyavoo *et al.,* 1997; Conti *et al.,* 1998; Fukumori *et al.,* 1998). On the other hand, several cellular proteins have been reported to associate with Vpr, such as a 41-kDa cytosolic protein that forms a complex with the glucocorticoid receptor protein (Refaeli *et al.,* 1995; Zhao *et al.,* 1994), an unidentified 180-kDa protein (Refaeli *et al.,* 1995), Sp1 (Wang *et al.,* 1995), TFIIB (Agostini *et al.,* 1996), uracil DNA glycosylase (Bouhamdan *et al.,* 1996), HHR23A (Withers-Ward *et al.,* 1997), p53 (Sawaya *et al.,* 1998), and a human 34-kDa mov34 homolog (Mahalingam *et al.,* 1998). Thus, the various roles of Vpr seem to involve modulation of the cellular environment via the interaction of Vpr with cellular partners.

A comparison of amino acid sequences and structural analysis reveal that a number of amino acid residues and structural motifs are conserved in the Vpr proteins of

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HIV-1, HIV-2, and SIV lentiviruses (Tristem *et al.,* 1992). Residues 17–34 seem very likely to form an amphipathic α-helical domain (containing five acidic and four leucine residues), as predicted by the Chou-Fasman algorithm (Mahalingam *et al.,* 1995a, 1995c). This domain appears to be critical for expression and stability of Vpr, as well as for incorporation into virions and nuclear localization (Mahalingam *et al.,* 1995a, 1995c; Yao *et al.,* 1995). A region spanning amino acids 46 through 74 is also predicted to have a helical structure, although with a significantly lower hydrophobic moment (Mahalingam *et al.,* 1995a), and it contains a determinant that is involved in the translocation to the nucleus of the preintegration complex in nondividing cells (Nie *et al.,* 1998). Another unique domain lies within amino acids 60–81. This region is rich in Ile/Leu residues and is known as the leucine zipper-like domain (Mahalingam *et al.,* 1997a; Wang *et al.,* 1996; Zhao *et al.,* 1994). This structure is involved in the nuclear localization of Vpr and in the specific interaction with a host cellular protein that is important for the function of Vpr (Mahalingam *et al.,* 1997a; Zhao *et al.,* 1994). The carboxy-terminal 20-amino-acid arginine-rich tail, which contains a cryptic nuclear localization signal (NLS), greatly impairs the nuclear localization of Vpr (Lu *et al.,* 1993; Mahalingam *et al.,* 1997a; Yao *et al.,* 1995; Zhao *et al.,* 1994; Zhou *et al.,* 1998). Truncation of or amino acid substitutions in this region result in failure to induce cell cycle arrest (Di Marzio *et al.,* 1995; Mahalingam *et al.,* 1997a; Zhou *et al.,* 1998). Thus, it appears that the carboxy-terminal region of Vpr is also required for induction of cell cycle arrest.

We found recently that Vpr interfered with the proliferation of mouse NIH3T3 fibroblasts but failed to induce the G_2 arrest of these same cells (Nishino *et al.,* 1997). Our findings suggested strongly that Vpr might retard cell proliferation via a novel pathway that is distinct from G_2 arrest. However, the mechanism for such retardation, remained obscure. It was also unclear whether the same phenomenon might occur in other established cell lines, in which the expression of Vpr can induce G_2 arrest. To examine these issues, we chose HeLa cells as a model cell line in which Vpr can induce G_2 arrest. We identified a truncated form of Vpr, designated C81, that lacked the carboxy-terminal arginine-rich region and failed to induce G_2 arrest in HeLa cells but retained the ability to interfere with the proliferation of these cells. Furthermore, results with variants of C81 with mutations within the leucine zipper-like domain, designed to disrupt the leucine zipper structure, suggested that the pathway utilized by Vpr for G_2 arrest might be distinct from the pathway, whereby C81 retards cell proliferation without G_2 arrest. Thus, we were able to demonstrate that Vpr can interfere with cell growth by at least two different mechanisms, each of which involves a different pathway.

RESULTS

Construction of plasmids and expression of Vpr deletion mutants. Vpr has a number of different functions during the life cycle of HIV-1, including nuclear import and the induction of cell cycle arrest at the G_2 phase. The various biological activities of Vpr are correlated with specific structural features of the protein (Di Marzio *et al.,* 1995; Mahalingam *et al.,* 1997a, 1995a, 1995b, 1995c; Piller *et al.,* 1996; Wang *et al.,* 1996; Yao *et al.,* 1995; Zhao *et al.,* 1994). As shown in Fig. 1A, Vpr can be divided into four putative structural regions on the basis of its amino acid sequence: (1) the first α-helical domain, extending from amino acids 17 to 34; (2) the second α-helical domain, extending from amino acids 46 to 74; (3) the leucine zipper-like domain, extending from amino acids 60 to 81; and (4) the arginine-rich carboxy-terminal domain (Lu *et al.,* 1993; Yao *et al.,* 1995; Zhou *et al.,* 1998). In order to identify the region(s) involved in the ability of Vpr to retard cell growth, we constructed cDNAs for Vpr mutants with deletion of each putative domain and then subcloned the cDNAs downstream of the SRα promoter in the expression vector pME18Neo (Fig. 1A).

We examined the effects of the deletion of each putative domain by transiently transfecting HeLa cells with an expression vector that encoded wild-type or mutant Vpr, constructed by using the primers shown in Table 1. To facilitate the assay, the wild-type and mutant proteins were given an amino terminal Flag tag with the sequence NH_2-Met-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (Fig. 1A). At 36 h after transfection, we examined the expression of Vpr by Western blotting analysis with MAb M2, which recognizes the Flag tag (Fig. 1C). We observed single bands of proteins with apparent molecular masses consistent with the predicted sequences in the analysis of HeLa cells that had been transfected with pME18Neo that encoded wild-type Vpr, Vpr lacking 15 amino acids of the arginine-rich carboxy-terminal region (C81), Vpr with deletion of the first α-helical domain (Δ17–34), and amino-terminally truncated Vpr that lacked 17 or 35 amino acids (N17 and N35, respectively). The C74 mutant, with a deletion of 22 amino acids at the carboxyl terminus, was expressed at levels significantly lower than those of the wild-type Vpr. By contrast, no specific bands were detected in the analysis of HeLa cells that had been transfected with pME18Neo that encoded a carboxy-terminally truncated Vpr that lacked 38 amino acids (C59), Vpr with deletion of the second α-helical domain or the leucine zipper-like domain (Δ46–74 and Δ60–81, respectively), or an amino-terminally truncated Vpr that lacked 60 amino acids (N60), as well as the negative control vector pME18Neo-Flag. Absence of detectable mutant Vpr proteins was presumably due to the decreased stability of these proteins. In addition, all transfections of the four vector plasmids in amounts up to 100 μg of transfected plasmid were neg-

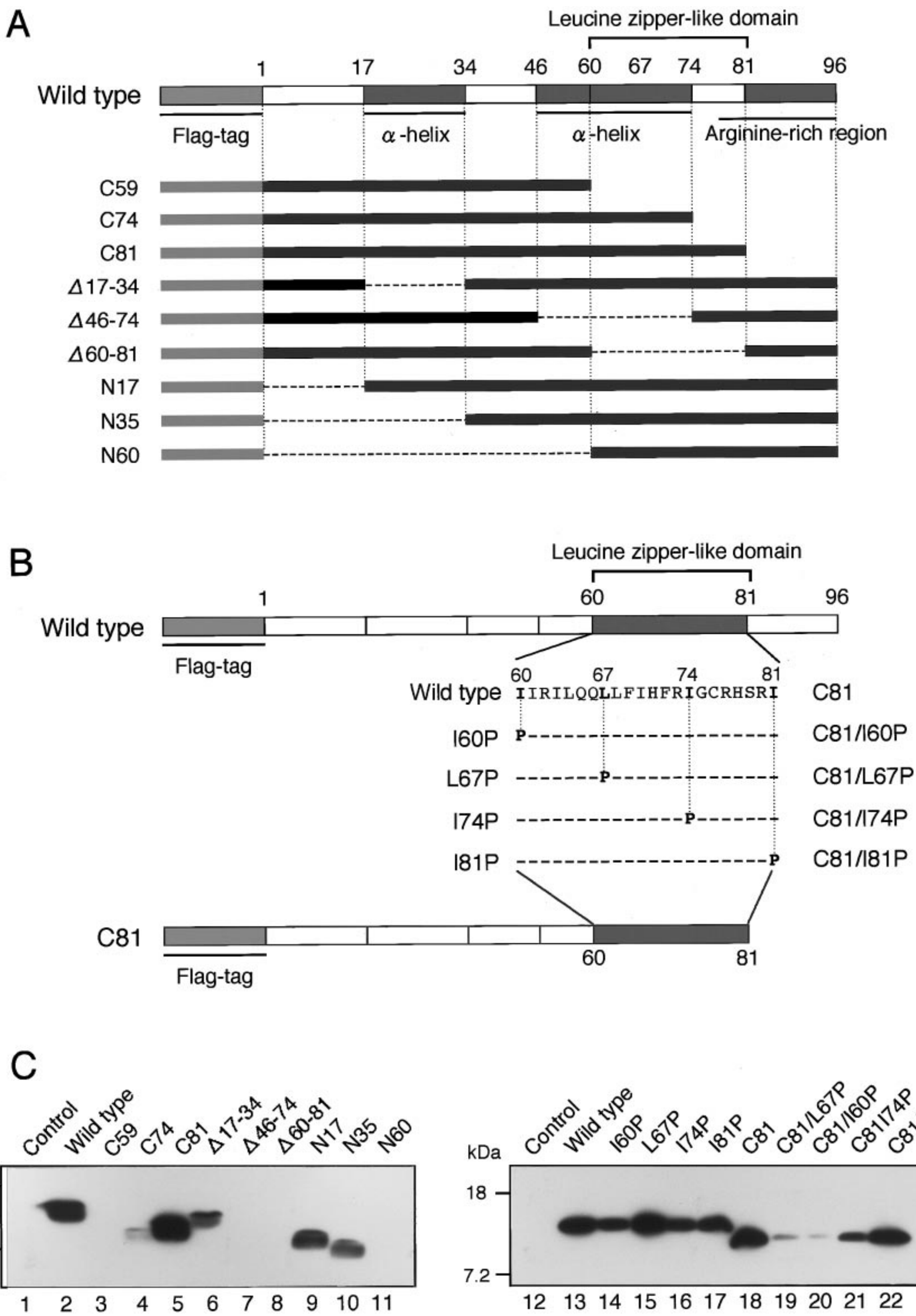

FIG. 1. Construction and expression of mutant Vpr proteins. (A) Plasmids containing cDNA for the deleted mutant forms of Vpr were generated by PCR from HIV-1_{NL4-3}. The positions of the predicted first α-helical domain, second α-helical domain, leucine zipper-like domain, and arginine-rich carboxy-terminal domain are indicated. The regions of the Vpr generated by each construct are shown in black; grey showing represents the Flag-tag. (B) The mutations introduced within the leucine zipper-like domain of wild-type Vpr and C81. The sequences of the leucine zipper-like domain (represented by dark shaded) are shown in the single-letter amino acid code and the locations of four Ile/Leu residues are indicated. The sequences of the mutant form of Vpr used in this study are indicated under the wild-type sequence, which was derived from HIV-1_{NL4-3}. (C) Western blotting of mutant Vpr proteins. Cells were cotransfected with pME18Neo that encoded Flag-tagged wild-type Vpr (lanes 2 and 13), C59 (lane 3), C74 (lane 4), C81 (lane 5), Δ17–34 (lane 6), Δ46–74 (lane 7), Δ60–81 (lane 8), N17 (lane 9), N35 (lane 10), N60 (lane 11), I60P (lane 14), L67P (lane 15), I74P (lane 16), I81P (lane 17), C81 (lane 18), C81/I60P (lane 19), C81/L67P (lane 20), C81/I74P (lane 21), or C81/I81P (lane 22) or the control pME18Neo-Flag (lanes 1 and 12) together with the pSV-β-Galactosidase plasmid. Transfected cells were harvested 36 h after transfection. Same cells were subjected to an assay of β-galactosidase activity and the rest were lysed. Lysates with equal β-galactosidase activity were subjected to Western blotting with the Flag-specific MAb M2. Positions of the molecular mass markers are indicated.

TABLE 1

Oligonucleotide Primers for Generation of Vpr Mutants[a]

Mutant	Forward primer	Restriction endonuclease	Reverse primer	Restriction endonuclease
Vpr	5′-GAAGATATCCGAACAAGCCCCCAGAAGAC-3′	*Eco*RV	5′-GCTCTAGATTGGTACAAGCAGTTTTAGGC-3′	*Xba*I
C59	5′-GAAGATATCCGAACAAGCCCCCAGAAGAC-3′	*Eco*RV	5′-TCTCTAGATCAGGCTTCCACTCCTGCCCA-3′	*Xba*I
C74	5′-GAAGATATCCGAACAAGCCCCCAGAAGAC-3′	*Eco*RV	5′-GGGTCTAGATCAAATTCTGAAATGGATAAA-3′	*Xba*I
C81	5′-GAAGATATCCGAACAAGCCCCCAGAAGAC-3′	*Eco*RV	5′-GGTCTAGATCATATTCTGCTATGTCGACAC-3′	*Xba*I
Δ17–34	5′-CCTAGGATATGGCTCCATAA-3′		5′-ATTGTATGGCTCCCTCTGTG-3′	
Δ46–74	5′-CGGCATAGCAGAATAGGCGTT-3′	*Nae*I	5′-GCACCCATGTTGTCCTAAGTTATG-3′	*Nae*I
Δ60–81	5′-GGAAGCCGGCGTTACTCGACAGAGGA-3′	*Nae*I	5′-ACTCCTGCCCACGTGTCCCCGTAAGTTTC-3′	*Pma*CI
N17	5′-CCGGATATCTGAATGGACACTAGAGCTTT-3′	*Eco*RV	5′-GCTCTAGATTGGTACAAGCAGTTTTAGGC-3′	*Xba*I
N35	5′-CCGGATATCTCCTAGGATATGGCTCCATA-3′	*Eco*RV	5′-GCTCTAGATTGGTACAAGCAGTTTTAGGC-3′	*Xba*I
N60	5′-CCGGATATCCATAATAAGAATTCTGCAAC-3′	*Eco*RV	5′-GCTCTAGATTGGTACAAGCAGTTTTAGGC-3′	*Xba*I
I60P	5′-GGAGTGGAAGCCCCAATTCGAATTCTGCAAC-3′	*Nsp*V	5′-TGCCCAAGTATCCCCGTAAG-3′	
L67P	5′-AAGAATTCTGCAGCAGCCACTGTTTATCCA-3′	*Pst*I	5′-TTATTATGGCTTCCACTCCTGCCCAAGTATCCCCGTAAG-3′	
I74P	5′-GGGTGTCGACATAGCAGAATAGGAGT-3′	*Sma*I	5′-GGGTCTGAAATGGATAAACAGCAG-3′	*Sma*I
I81P	5′-TCGACATAGCAGGCCTGGCGTTACTCGA-3′	*Aat*I	5′-CACCCAATTCTGAAATGGAT-3′	
C81/I81P	5′-GAAGATATCCGAACAAGCCCCCAGAAGAC-3′	*Eco*RV	5′-GGTCTAGATCAAGGCCTGCTATGTCGACAC-3′ *Xba*I *Aat*I	*Xba*I, *Aat*I

[a] The underlined sequences are restriction site of endonuclease.

ative (data not shown). Moreover, no specific bands were detected when the analysis was performed with normal mouse serum (data not shown). Thus, these results indicate that the putative second α-helical domain that extends from amino acids 46 to 74 might be involved in expression of Vpr. In subsequent experiments, we used only mutant forms of Vpr whose expression was detectable by Western blotting analysis.

The C81 mutant protein retarded cell growth independently of any induction of G_2 arrest. To determine which of the putative domains of Vpr is required for G_2 arrest, we cotransfected HeLa cells with pME18Neo that encoded wild-type, C74, C81, Δ17–34, N17, or N35 Vpr or the control pME18Neo-Flag together with a small amount of a GFP expression vector, pEGFP-N1, which served as a marker plasmid. Forty-eight hours after transfection, we stained the cells with propidium iodide and then determined the distribution within the cell cycle transfected cells using a FACScan to measure the DNA content of GFP-expressing cells (Table 2 and Fig. 2). In the case of cells transfected with the expression vector that encoded wild-type Vpr, there was a dramatic increase in the proportion of cells in the G_2/M phase of the cell cycle as compared with cells transfected with the control vector pME18Neo-Flag. By contrast, all transfections with vectors that encoded our deletion mutants were unable to arrest the cell cycle at the G_2/M phase. However, one of five mutants, C81 caused a block of the cell cycle at the G_1 phase; only 3.1% of the cells were in G_2/M phase, while 76.7% of the cells were in G_1.

To determine in further detail the effects of the deletion in each putative domain in Vpr on cell proliferation, we performed a colony formation assay. HeLa cells were transfected with pME18Neo, which contains a neomycin-resistance marker, that encoded wild-type and mutant Vpr proteins and then, 48 h after transfection, transfected cells were transferred to selective medium that contained G418. Twelve days later, surviving colonies were counted to assess the growth-inhibitory effects of the various proteins and the relative number of colonies was calculated as the actual number of colonies/relative β-Gal activity to normalize the efficiency (Table 3). The percentage inhibition of the maximal proliferation in HeLa cells is also shown for each protein in Table 3. Transfection of HeLa cells with pME18Neo-Fvpr resulted in a dramatically reduced growth rate and prevented the establishment of drug-resistant cells, as compared with transfection with the control vector pME18Neo-Flag. This result suggested that Vpr can both induce the arrest of HeLa cells in the G_2 phase and block cell growth. Moreover, C81, which lacked the ability to induce G_2 arrest and

TABLE 2

Cell Cycle Arrest Activity in HeLa Cells Expressing Wild-Type and Deletion Mutant Vpr Proteins[a]

Mutant	Relative G_2/M:G_1 ratio	G_2 arrest
Control	0.29[b]	–[c]
Wild type	1.00	+
C74	0.29	–
C81	0.04	–
Δ17–34	0.38	–
N17	0.36	–
N35	0.33	–

[a] HeLa cells were transfected with pME18Neo that encodes Flag-tagged wild-type Vpr, mutant or control pME18Neo-Flag together with the green fluorescent protein (GFP) expression vector, pEGFP-N1. Then, 48 h after transfection, DNA content of cells was determined as described in the legend of Fig. 2.

[b] Values were defined in each experiment by setting the G_2/M:G_1 ratios for cells that expressed wild-type Vpr to 1.0. The results represent the mean of three independent experiments of each mutant.

[c] +, Full G_2 arrest; –, no G_2 arrest.

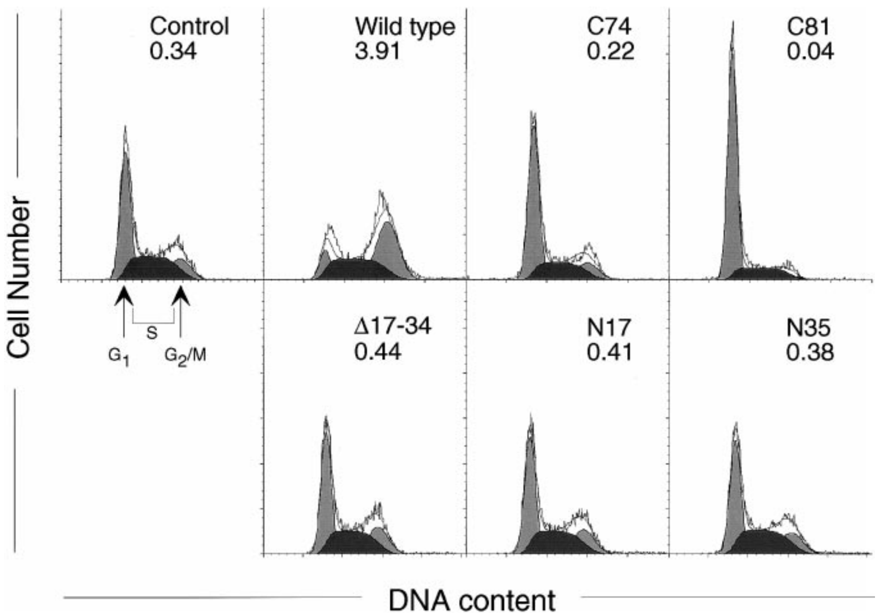

FIG. 2. DNA content of HeLa cells that expressed Flag-tagged wild-type Vpr and deletion mutant forms of Vpr. HeLa cells were transfected with pME18Neo that encoded Flag-tagged wild-type, C74, C81, Δ17–34, N17, N35, or the control pME18Neo-Flag together with a GFP expression vector, pEGFP-N1. Then, 48 h after transfection, cells were harvested for analysis of DNA content and stained with propidium iodide. Cells that were GFP-positive were analyzed by flow cytometry using the Lysis II for acquisition and ModFit LT for quantitative analysis of DNA content. Arrows indicate peaks of cells at the G_1 and G_2/M phases. The G_2/M:G_1 ratio is indicated at the upper right of each graph.

gained capacity to lead G_1 arrest, retained the cell growth arrest activity, albeit to a lesser extent (approximately 65%) than wild-type Vpr. All the remaining mutants tested that had failed to induce the G_2 arrest also failed to suppress the growth of HeLa cells.

TABLE 3

Proliferation of Colony Forming Cells Expressing Wild-Type and Deletion Mutant Vpr Proteins[a]

Mutant	Relative number of colonies[b]	Inhibition of growth[c] (%)
Control	527.7 ± 47.3[d]	0 ± 10
Vpr	40.8 ± 7.1	100 ± 1
C74	544.0 ± 19.9	−3 ± 4
C81	358.7 ± 6.0	35 ± 1
Δ17–34	624.6 ± 37.0	−20 ± 8
N17	738.4 ± 39.6	−44 ± 8
N35	691.7 ± 83.6	−34 ± 17

[a] HeLa cells were cotransfected with pME18Neo that encoded Flag-tagged wild-type Vpr, C74, C81, Δ17–34, N17 or N35 or the control pME18Neo-Flag together with a pSV-β-Galactosidase plasmid. Then, 48 h after transfection, cells were harvested. Some cells were subjected to an assay of β-galactosidase activity, and the rest were replated in selective medium that contained G418.

[b] The data shown present the relative number of drug-resistant colonies counted twelve days after drug selection. The relative number of colonies was calculated as actual number of colonies/relative β-Gal activity.

[c] The blocking of colony formation by each mutant was normalized by reference to parallel transfections with pME18Neo-Fvpr or a control pME18Neo-Flag to 100 and 0% blocking, respectively.

[d] Mean ± SD.

These results suggest that C81 mutant can retard cell growth via a mechanism different from that by which it arrests cells at the G_2 phase of the cell cycle. Thus, it seems likely that Vpr protein has two functions in cell growth: it can suppress growth without G_2 arrest of the cell cycle and it can also induce G_2 arrest. The former effect may correlate with a G_1 arrest of cell cycle.

Mutagenesis of Ile/Leu residues in the leucine zipper-like domain in wild-type Vpr and C81. To determine whether the Vpr-induced G_2 arrest of the cell cycle and the C81-induced suppression of growth without G_2 arrest are regulated by an independent pathway or the same pathway, we focused on the leucine zipper-like domain from amino acids 60 to 81 of Vpr. This region appears to be able to form a typical leucine zipper-like structure of the type found in a variety of transcription factors, which can provide a site for direct contact with other proteins (Wang *et al.,* 1996). The amino acid sequences of this region of Vpr derived from various isolates of HIV-1, HIV-2, and SIV are compared (data not shown). Four Ile/Leu residues at positions 60, 67, 74, and 81 are almost fully conserved and they are arranged similarly in the leucine zippers identified in the transcription factors from 20 laboratory isolates of HIV-1. However, the Ile/Leu residues at positions 67 and 74 but not at positions 60 and 81 are replaced by other amino acids, such as Ala, Gly, Gln, and Ser, in isolates of HIV-2 and SIV. Thus, conservation of the four Ile/Leu residues in the leucine zipper-like domain is characteristic of HIV-1. Furthermore, the leucine zipper-like domain in Vpr might contribute to the pathogenicity of HIV-1 since several groups of investigators (Mahalingam *et al.,* 1997a; Wang *et al.,* 1996, 1995) have shown that a leucine zipper-like domain might be an important functional determinant of Vpr of HIV.

To compare the effects of substitutions within the leucine zipper-like domain on the two growth-suppressing activities of Vpr, we generated derivatives of pME18Neo that encoded the mutant forms of wild-type Vpr and C81 with replacement of each Ile/Leu residue by Pro to introduce changes in the leucine zipper-like domain of wild-type (I60P, L67P, I74P, and I81P) and C81 (C81/I60P, C81/L67P, C81/I74P, and C81/I81P) Vpr proteins (Fig. 1B). Western blotting analysis with MAb M2 revealed that each protein with a site-specific mutation was expressed at detectable levels in the corresponding transfected cells (Fig. 1C).

The C81 mutant exploits a novel pathway to retard cell growth that is independent of the pathway that leads to G_2 arrest. We analyzed the results of transfections with our series of Vpr expression vectors to define the Ile/Leu residues that are required for G_2 arrest, as shown schematically in Fig. 1B. The DNA content of HeLa cells that had been transiently transfected with each derivative of pME18Neo that encoded Vpr with a site-specific mutation was analyzed by flow cytometry (Fig. 3A). After transient transfection with I74P and I81P expression vectors, no HeLa cells were arrested at the G_2 phase, whereas about 17.7 and 17.2% of cells transfected with I60P and L67P expression vectors, respectively, were arrested at the G_2 phase, as compared to cells that expressed wild-type Vpr. Likewise, colony formation assay indicated that after transfection of HeLa cells with I74P and I81P expression vectors, these cells did not show growth inhibition in contrast to I60P and L67P expression vectors (Fig. 3B). These results clearly indicate that the leucine zipper-like domain, and in particular the Ile residues at positions 74 and 81, were indispensable for induction of cell cycle arrest at the G_2 phase and subsequent growth arrest (Table 4).

In order to evaluate the significance of the leucine zipper-like domain in C81 in preventing cell proliferation, we transiently transfected HeLa cells with pME18Neo that encoded wild-type Vpr, C81, and C81 with site-specific mutations (C81/I60P, C81/L67P, C81/I74P, and C81/I81P). After transfection and overnight incubation, the cells were incubated with [^{3}H]thymidine and incorporation of the radiolabel into the cells was measured (Fig. 4). The suppression the cell growth of HeLa cells transfected with the C81 expression vector was stronger than that of cells transfected with the wild-type Vpr expression vector (incorporation of approximately 7×10^3 and 1.6×10^4 cpm of [^{3}H]thymidine, respectively, in the case of C81 and wild-type Vpr). All of the four site-specific mutations decreased the growth-suppressive activity of C81. In particular, substitutions at Ile60, Leu67, and Ile74 completely eliminated this activity. After substitution of Ile81 by Pro, mutated C81 retained approximately 40% of the activity of C81 itself. Furthermore, no recovery of the ability to induce G_2 arrest was observed in the case of any of the mutant forms of C81 with site-specific mutations (data not shown). These results suggest that the leucine zipper-like domain might be essential not only for G_2 arrest but also for the suppression of growth without G_2 arrest and, moreover, that the Ile/Leu residues at positions 60, 67, and 74 might be important for the growth arrest induced by C81 (Table 4).

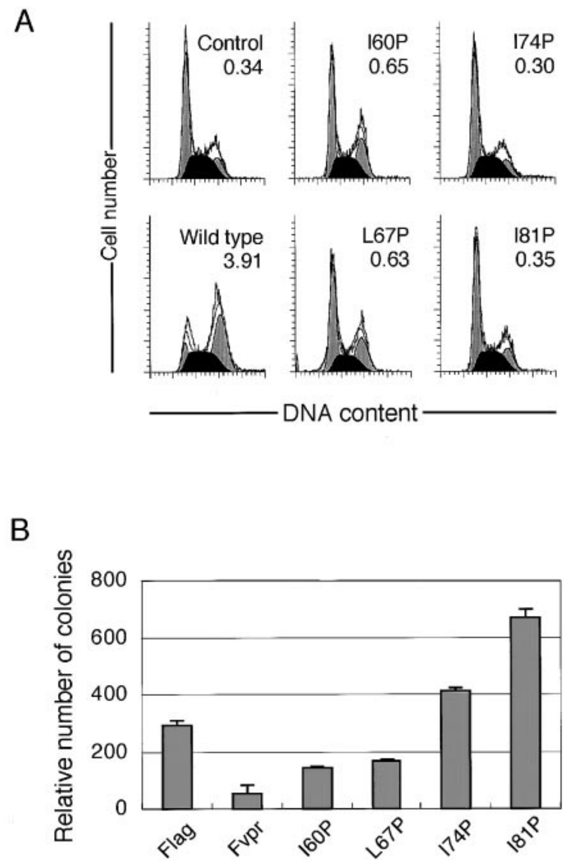

FIG. 3. Analysis of the cell cycle and the proliferation of colony formation of HeLa cells expressing the substitution mutant Vpr proteins. HeLa cells were transfected with pME18Neo encoding Flag-tagged wild-type Vpr, I60P, L67P, I74P, or I81P or the control pME18Neo-Flag together with a GFP expression vector, pEGFP-N1 (A), or a pSV-β-Galactosidase plasmid (B). (A) At 48 h posttransfection, DNA content of the cells was determined by flow cytometry as described in the legend of Fig. 2. G_2/M:G_1 ratios are indicated at the upper right of each graph. (B) Twelve days later, surviving colonies were counted and then the relative number of colonies was calculated as actual number of colonies/relative β-Gal activity as described in Table 3. Each column and error bar represent the mean ± SD of results from three samples.

As summarized in Table 4, induction of G_2 arrest by Vpr and the retardation of growth by C81 appeared to depend on different residues in the leucine zipper-like domain of Vpr. Thus, our observations strongly suggest that G_2 arrest induced by wild-type Vpr and the C81-induced

TABLE 4

Effects of Substitution of Ile/Leu Residues within the Leucine Zipper-like Domain of Wild-Type Vpr and C81 on the Activities of These Proteins

		Activity[a]	
		G_2 arrest	Suppression of growth, independent of G_2 arrest
Wild-type Vpr		+++	Unknown[b]
Substitution at position	60	+	Unknown
	67	+	Unknown
	74	−	−
	81	−	−
C81		−	+++
Substitution at position	60	−	−
	67	−	−
	74	−	−
	81	−	++

[a] Extent of activity: −, none; +, slight; ++, moderate; +++, marked.
[b] Whether the expression of Vpr and mutants retard the cell proliferation independently of G_2 arrest is unknown.

suppression of growth might involve different mechanisms or pathways.

DISCUSSION

In a previous study, we have found that Vpr interfered with the proliferation of mouse NIH3T3 cells but failed to induce the G_2 arrest of these cells (Nishino *et al.,* 1997). This finding suggested strongly that Vpr might be able to retard cell proliferation via a pathway distinct from the pathway that leads to G_2 arrest. In this study, we confirmed that the phenomenon observed in NIH3T3 cells occurred in another established cell line, in which the expression of Vpr can induce G_2 arrest. Using C81, which lacked the carboxy-terminal arginine-rich region of Vpr, and HeLa cells, in which Vpr induces G_2 arrest, we obtained evidence that Vpr can interfere with cell growth via two distinct pathways in the same cells, namely, growth suppression without G_2 arrest and growth suppression with G_2 arrest are as follows: (i) Vpr induce G_2 arrest and blocked the growth of HeLa cells. Flow cytometric analysis indicated that C81 completely failed to induce G_2 arrest in HeLa cells, even though the results of the colony formation assay and the incorporation of [^{3}H]thymidine showed that C81 inhibited the proliferation of these same cells; and (ii) mutation analysis indicated that the induction of G_2 arrest depended particularly on the Ile residues at positions 74 and 81 in the putative leucine zipper-like domain, whereas the suppression of C81 of cell proliferation without G_2 arrest involved Ile/Leu residues at positions 60, 67, and 74. Our data indicated clearly that the functional residues required for the two different activities were different from one another. Collectively, our results demonstrate that induction of G_2 arrest and growth suppression without G_2 arrest are independent functions of Vpr. Indeed, our present result suggests that C81-induced suppression of growth may result in G_1 arrest. Therefore, it appears that C81 is an important tool for characterization of the mechanism of growth suppression by Vpr without G_2 arrest, and, in addition, C81 appears to provide the optimal protein conformation for this function of Vpr.

Rogel *et al.* (1995) indicated that HIV-1 virions that contain an intact *vpr* gene are unable to establish the chronic infection of T-cells. Furthermore, transfection of a *vpr* gene together with the neomycin-resistance gene in the absence of other viral genes was found to decrease the formation of geneticin-resistant colonies for 10 to 15 days posttransfection (Planelles *et al.,* 1995). It has been shown recently that expression of Vpr in several lines of human tumor cells after transfection results in a marked reduction in colony formation *in vitro* and a significantly reduced ability to form tumors *in vivo* (Mahalingam *et al.,* 1997b). These results support the results of our colony formation assay, namely, that transfection of HeLa cells with a wild-type Vpr expression vector resulted in cells with a dramatically reduced growth rate and prevented the establishment of the drug-resistant cells for 12 days posttransfection. Thus, our data and those of others indicate that Vpr might act to prevent cell proliferation over a relatively long period. We demonstrated that G_2 arrest is not required for C81-induced suppression of growth. Likewise, our previous report showed that Vpr retards the growth of NIH3T3 cells independently of the

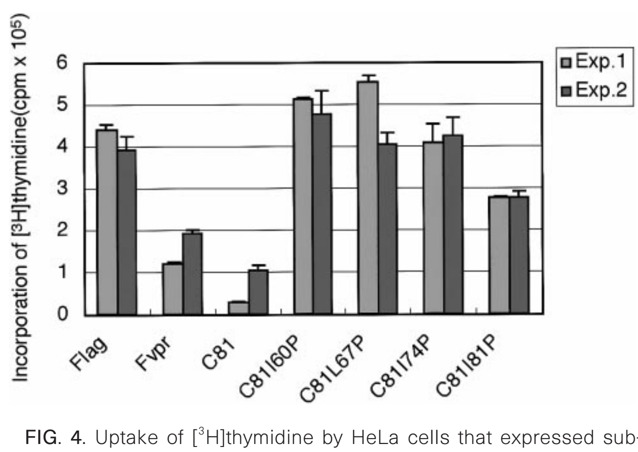

FIG. 4. Uptake of [^{3}H]thymidine by HeLa cells that expressed substitution mutant Vpr with site-specific. HeLa cells were transfected with pME18Neo that encoded Flag-tagged wild-type Vpr, C81, C81/I60P, C81/L67P, C81/I74P, or C81/I81P or the control pME18Neo-Flag together with a pSV-β-Galactosidase plasmid. Twelve hours after transfection, some cells were assayed for β-Gal activity and the rest were replated at 1×10^5 cells/well in 24-well flat-bottomed plates. After a 30-h incubation, cells were incubated for 12 h with 0.5 μCi of [^{3}H]thymidine and then harvested on glass fiber filters. The incorporation of radioactivity was determined by liquid scintillation counting and incorporation of [^{3}H]thymidine was calculated as radioactivity/β-Gal activity. Each column and error bar represent the mean ± SD of results from four samples in two independent experiments.

induction of G_2 arrest (Nishino *et al.,* 1997). These results strongly suggest that wild-type Vpr might function to block cell proliferation without the induction of G_2 arrest, as does the C81 mutant protein. However, as in the case of C81, it remains unclear whether the expression of wild-type Vpr in cells leads to the suppression of cell growth independently of G_2 arrest. One hypothesis suggested by our results is that the expression of Vpr at high levels in cells immediately and strongly induces G_2 arrest and the induction of G_2 arrest overcomes other functions of Vpr. It is now essential to elucidate the mechanism of the growth-suppressive activity of C81 that does not involve the induction of G_2 arrest.

In the present study, we generated mutant forms of Vpr with deletion of each putative structural region, namely, the first α-helical domain, the second α-helical domain, the leucine zipper-like domain, and the arginine-rich carboxy-terminal domain. All of the mutant proteins failed to induce G_2 arrest. There are two possible explanations for these results. First, failure to induce G_2 arrest might have been due to altered structural conformations associated with the mutated Vpr proteins. Alternatively, each of the putative domains might play a pivotal role in induction of G_2 arrest. The hypothesis that the entire domain structure of Vpr is required for induction of G_2 arrest is supported by the following results. (i) In this study, proteins with site-specific mutations within the leucine zipper-like domain of Vpr, I74P, and I81P failed to induce G_2 arrest. (ii) Mahalingam *et al.* (1997a) reported that proteins with two missense mutations, A30L in the first α-helical domain and R80A in the carboxy-terminal domain, failed to induce G_2 arrest (Di Marzio *et al.,* 1995). (iii) Vpr from HXB2 (with an 18-amino-acid deletion at carboxyl terminus) induced neither cell cycle arrest nor an unusual morphological phenotype (Mahalingam *et al.,* 1997a).

Recently, it was reported that activation by Vpr of transcription of HIV-1 is correlated with the ability of Vpr to induce G_2 arrest (Felzien *et al.,* 1998; Goh *et al.,* 1998). In particular, Vpr was found to increase the production of virus by delaying cells at the point in the cell cycle at which the viral LTR is most active (Goh *et al.,* 1998). Moreover, stable expression of low levels of Vpr in host cells appears to act as a negative regulator of apoptosis in a line of human lymphoblastoid cells and represents an additional strategy for the persistence and spread of HIV (Conti *et al.,* 1998). Therefore, further study is required to define clearly whether the C81-induced suppression of cell growth without G_2 arrest regulated the replication of HIV-1.

MATERIALS AND METHODS

The construction of plasmids. HIV-1_{NL4-3} (Adachi *et al.,* 1986) *vpr* was amplified by PCR with primers indicated in Table 1 and it was subcloned between the *Xba*I and *Eco*RV sites of pBluescript II (SK^+) (Stratagene, La Jolla, CA) that encoded a Flag with the following amino acid sequence: NH_2-Met-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, to yield a pSK-Fvpr. The *Xho*I–*Not*I fragment containing *vpr* and the Flag sequence of pSK-Fvpr was further subcloned downstream of the SRα promoter in the expression vector pME18Neo (Tajima *et al.,* 1998) to yield a plasmid designated pME18Neo-Fvpr (Nishino *et al.,* 1997). In addition, pME18Neo-Flag, which contained only the Flag sequence, was constructed as a control vector (Nishino *et al.,* 1997). The *vpr* sequences with deletions, which encoded proteins designated C59, C74, C81, N17, N35, and N60, together with an amino-terminal Flag sequence, were amplified by PCR with pSK-Fvpr as template and the primers shown in Table 1. Each *Xho*I–*Not*I fragment containing a deleted *vpr* sequence plus the Flag-encoded sequence was then subcloned into pME18Neo. To generate the substitution mutants designated, I60P, L67P, I74P, and I81P, we introduced site-specific mutations into pSK-Fvpr by following the instructions in the manual provided with the ExSite PCR-based site-directed mutagenesis kit from Stratagene (Weiner *et al.,* 1994), as shown schematically in Fig. 1B. Leu or Ile codons in the leucine zipper-like domain were changed to Pro codons by long and accurate PCR (LA-PCR) with pSK-Fvpr as the template and the primers indicated in Table 1. The primers were designed to introduce the desired amino acids of specific sites, as well as specific site of restriction enzyme (Table 1). Each *Xho*I–*Not*I fragment, including the site-mutated *vpr* and Flag sequences, in pSK-Fvpr was excised and subcloned into pME18Neo. Similarly, cDNAs for Δ17–34, Δ46–74, and Δ60–81 were amplified by LA-PCR with pSK-Fvpr as template and the primers indicated in Table 1, and each the *Xho*I–*Not*I fragment was then subcloned into pME18Neo. To generate three substitution mutants of C81, namely, C81/I60P, C81/L67P, and C81/I74P, we excised the *Eco*RV–*Sal*I fragment that encoded from amino acids 1 to 75 of the Vpr protein from pSK-FI60P, pSK-FL67P, or pSK-FI74P, which corresponded to site-specific mutations in pSK-Fvpr. We introduced each fragment into pSK-FC81, and then each *Xho*I–*Not*I fragment that included the mutated *vpr* and Flag sequences was introduced into pME18Neo. To generate the C81/I81P mutant, the product of PCR was amplified by LA-PCR with pSK-FI81P as template and the primers indicated in Table 1. The product was subcloned between the *Eco*RV and *Xba*I sites of pSK-Fvpr, and then the *Xho*I–*Not*I fragment that included the mutated *vpr* and Flag sequence was excised and subcloned into pME18Neo. All constructs described above were verified by nucleotide sequencing by the dideoxy chain-termination method (Sanger *et al.,* 1977) with a *Bca*BEST dideoxy sequencing kit (Takara, Otsu, Japan).

pEGFP-N1 encodes a red-shifted variant of wild-type green fluorescent protein (GFP), which has been modified for brighter fluorescence (Cormack *et al.,* 1996). It was used for flow cytometry. pSV-β-Galactosidase encodes a bacterial β-galactosidase and was used for normalization of the efficiency of transfection.

Cell culture and transfection. Human cervical HeLa cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactive fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells (1×10^7) were transfected with 47.5 μg of expression vector and 2.5 μg of pSV-β-Galactosidase or 2.5 μg of pEGFP-N1 by electroporation in a 0.4-cm-diameter cuvette using a Bio-Rad gene pulsar (Richimond, CA) at 300 V and 975 μF.

Western blotting. Thirty-six hours after transfection, the expression of Vpr was examined by Western blotting analysis as described previously (Tajima *et al.,* 1998).

Analysis of the cell cycle. Forty-eight hours after transfection, cells were harvested, fixed in 1% formaldehyde and 70% ethanol, and then incubated in phosphate-buffered saline that contained propidium iodide (50 μg/ml), RNase A (50 μg/ml), and FCS (2%, v/v) for 60 min at room temperature. The fluorescence of 10,000 cells was analyzed on a FACScan system (Becton-Dickinson, Mountain View, CA) using the Lysis II software (Becton-Dickinson). Data are presented after gating to eliminate cells in which GFP emitted strong fluorescence. Ratios of numbers of cells in the G_1 and G_2/M phase (G_2/M:G_1 ratios) were calculated with ModFit LT software (Verity Software House, Topsham, ME).

Proliferation of colony-forming cells. Forty-eight hours after transfection, cells were harvested and divided into two portions. Some cells were subjected to an assay of β-Gal activity (β-Gal Reporter Gene Assay kit; Boehringer-Mannheim, Mannheim, Germany), and the rest were replated (5×10^5) in a 10-cm-diameter dish with 10 ml of growth medium that contained 1 mg/ml of G418 (Gibco BRL, Grand Island, NY). After 12 days, the G418-resistant colonies were fixed in methanol, stained with Giemsa solution, and counted. To normalize the efficiency of transfection, the number of colonies was calculated relative to the initial β-Gal activity.

Incorporation of [^{3}H]thymidine. Twelve hours after transfection, cells were replated at 1×10^5 cells/well in 24-well flat-bottomed plates. Then 30 h later, 0.5 μCi of [^{3}H]thymidine (NEN) was added to each well and incubation was continued for 8 h. The cells were harvested and collected on a glass fiber filter and the radioactivity, as counts per minutes, was determined by liquid scintillation counting. To normalize the efficiency of transfection, relative incorporation of [^{3}H]thymidine was calculated as radioactivity/β-Gal activity.

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