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Effect of Human Immunodeficiency Virus Type 1 Protein R (*vpr*) Gene Expression on Basic Cellular Function of Fission Yeast *Schizosaccharomyces pombe*

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The human immunodeficiency virus type 1 (HIV-1) Vpr protein affects cell morphology and prevents proliferation of human cells by induction of cell cycle G_2 arrest. In this study, we used the fission yeast *Schizosaccharomyces pombe* as a model system to investigate the cellular effects of HIV-1 *vpr* gene expression. The *vpr* gene was cloned into an inducible fission yeast gene expression vector and expressed in wild-type *S. pombe* cells, and using these cells, we were able to demonstrate the specific Vpr-induced effects by induction and suppression of *vpr* gene expression. Induction of HIV-1 *vpr* gene expression affected *S. pombe* at the colonial, cellular, and molecular levels. Specifically, Vpr induced small-colony formation, polymorphic cells, growth delay, and cell cycle G_2 arrest. Additionally, Vpr-induced G_2 arrest appeared to be independent of cell size and morphological changes. The cell cycle G_2 arrest correlated with increased phosphorylation of $p34^{cdc2}$, suggesting negative regulation of mitosis by HIV-1 Vpr. Treatment of Vpr-induced cells with a protein phosphatase inhibitor, okadaic acid, transiently suppressed cell cycle arrest and morphological changes. This observation implicates possible involvement of protein phosphatase(s) in the effects of Vpr. Together, these data showed that the HIV-1 Vpr-induced cellular changes in *S. pombe* are similar to those observed in human cells. Therefore, the *S. pombe* system is suited for further investigation of the HIV-1 *vpr* gene functions.

Human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr) is a small, 15-kDa, virion-associated regulatory protein (4, 22). Its function is required for efficient replication and to facilitate entry of viral nucleic acids into the nuclei of macrophages and monocytes (6, 10). Vpr was also shown to perturb host cellular functions. Specifically, Vpr alters cell morphology (20, 34) and prevents cell proliferation by arresting cells in the G_2 phase of the cell cycle (9, 15, 34, 35).

The fission yeast *Schizosaccharomyces pombe* is a monocellular eukaryote that can be readily grown and manipulated in the laboratory by a variety of sophisticated methodologies. It shares many molecular, genetic, and biochemical features with higher eukaryotic cells, making it a particularly useful model to study the function and regulation of genes from more complex multicellular organisms (for a recent review, see reference 37). *S. pombe* is exceptionally well suited for the study of cross-species gene activities involved in basic cellular functions such as cell cycle control. This is based on the fact that *S. pombe* has a typical cell cycle and divides in a binary fashion which resembles that of human cells. The conservation of gene activity in cell cycle control between human and *S. pombe* cells can be clearly demonstrated by functional complementation of a number of *S. pombe* mutants by human cognates, including cell cycle control genes *cdc2*, *cdc13*, *cdc25*, and *wee1* (13, 19, 29, 32). The *S. pombe cdc2* and *cdc13* genes encode $p34^{cdc2}$ and cyclin B. Association of these two gene products is required for entry of cells into mitosis in all eukaryotes (27, 30). Interruption of $p34^{cdc2}$-cyclin B complex formation often blocks G_2-M phase transition and results in cell cycle G_2 arrest. Recent studies showed that Vpr affects specifically cyclin B-associated $p34^{cdc2}$ (2) and induces G_2 arrest by preventing the activation of the $p34^{cdc2}$-cyclin B complex (9, 15, 34), suggesting that the effect of Vpr on cell cycle control must be modulated through a common cellular process. Therefore, *S. pombe*, as a model system, should be particularly useful to investigate the molecular mechanisms by which the HIV-1 *vpr* gene modulates basic cellular functions. In this report, the effects of HIV-1 *vpr* gene expression on basic cellular functions of *S. pombe* are presented.

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MATERIALS AND METHODS

Yeast strains and media. A wild-type *S. pombe* strain, SP223 (h⁻ *ade-216 leu1-32 ura4-294*), was regularly maintained in YEA medium (0.5% Difco yeast extract, 3% glucose, and adenine [75 μg/ml]). Selection media for growth of *S. pombe* cells containing the expression vectors or recombinant constructs included standard Edinburgh minimal medium (EMM) and low glucose (0.4% glucose) and low nitrogen (2.5 μM NH_4Cl) media supplemented with uracil, adenine, and leucine, all at 75 μg/ml, as needed (7). Thiamine, prepared in distilled water as a 20 mM stock solution of thiamine hydrochloride, was added to the medium at a final concentration of 20 μM. All liquid cultures were grown at 30°C, with constant shaking at 200 rpm. Agar plates were incubated at 30°C for 3 to 4 days to obtain individual colonies.

Molecular cloning of the *vpr* gene into an *S. pombe* expression vector. The *vpr* gene of the HIV-1 viral isolate NL4-3 was cloned into a fission yeast expression vector, pREP1N (24). This vector contains an inducible *nmt1* (no message in thiamine) promoter, which can be repressed or induced in the presence or absence of thiamine, respectively (3, 23). Insertion of the *vpr* gene into pREP1N was confirmed by extracting DNA from individual *Escherichia coli* colonies containing the plasmid and subsequently analyzing the DNA by restriction mapping and PCR. The complete wild-type nucleotide sequence of the *vpr* gene was confirmed by DNA sequencing. One clone was chosen and designated pREP1N-*vpr*.

Measurement of cell cycle G_2 arrest by flow cytometry and septation index analyses. The procedure used to prepare *S. pombe* cells for flow cytometry analyses was modified from that of Alfa et al. (1). Briefly, cells containing pREP1N-*vpr* were grown to stationary phase in 5 ml of EMM containing thiamine, with constant shaking at 30°C. A 1-ml aliquot of culture was collected, washed three times with distilled water to remove thiamine, and reinoculated into 5 ml of low nitrogen or low glucose medium at a concentration of 5×10^5 cells per ml with or without thiamine. Cells were collected at approximately 48 h

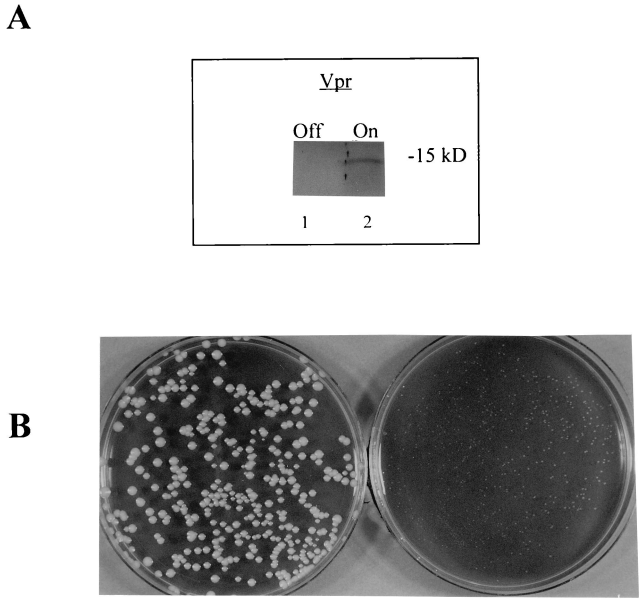

FIG. 1. HIV-1 *vpr* gene expression and its effect on colony formation. (A) Immunoblot analysis of HIV-1 *vpr* expression in *S. pombe*. Vpr was detected with the HIV-1 BH10 Vpr antiserum (22). Cell lysates were collected after 34 h of incubation with (lane 1; *vpr* off) and without (lane 2; *vpr* on) thiamine. (B) HIV-1 Vpr-induced small-colony formation and color change. The wild-type *S. pombe* cells containing pREP1N-*vpr* were plated onto the minimal selection EMM agar plate with (left-hand plate; *vpr* off) and without (right-hand plate *vpr* on) thiamine.

and fixed with 7 ml of 95% ethanol. Before flow cytometry analysis, cells were treated with RNase A (5 μg/ml) in 50 mM sodium citrate (pH 7.0) and stained with propidium iodine (catalog no. P 4170; Sigma). The level of fluorescence in individual cells was analyzed on a FACScan (Becton Dickinson) using LySys II software (Becton Dickinson). Ten thousand events were collected, and the level of fluorescence corresponding to cells in G_1 and G_2 was determined as the FL2 parameter (FL-2 measures the amount of propidium iodine fluorescence emitted through a 585-nm band-pass filter).

Aliquots were taken at appropriate times and fixed in preparation for septation index analysis. *S. pombe* cells were stained with calcofluor white M2R (F6259; Sigma) to enhance the visualization of the septa. After staining, the number of cells passing mitosis were estimated in triplicate by counting the proportion of septated cells in random fields of approximately 500 cells.

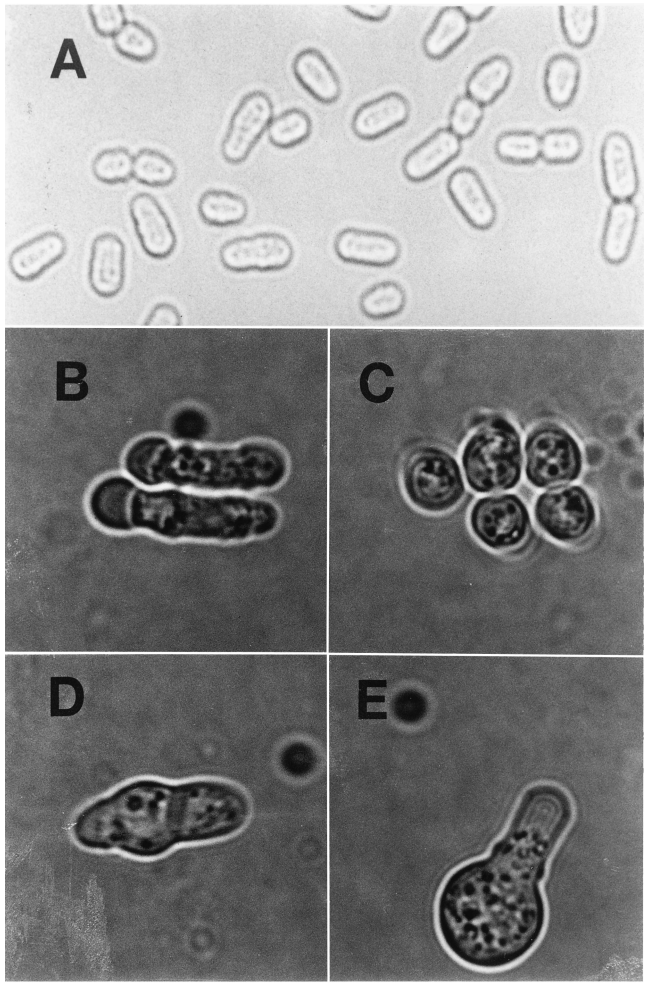

FIG. 2. Polymorphism of *S. pombe* cells induced by HIV-1 Vpr. (A) *vpr*-off, normal cell morphology. Induction of *vpr* expression induces polymorphic cellular changes, including elongated (B), spherical (C), irregular (D) and balloon-like (E) cells.

RESULTS

Expression of the *vpr* gene in *S. pombe* cells. The pREP1N-*vpr* plasmid was transformed into the wild-type SP223 *S. pombe* cells by a lithium acetate DNA transformation procedure (16, 31). The transformed cells were plated onto the selection EMM agar plates with thiamine (*vpr* gene expression was repressed) and without thiamine (*vpr* gene was constitutively expressed). *S. pombe* cells containing the transforming plasmid were selected for their ability to grow in leucine-free medium. Immunoblot analysis was performed to confirm Vpr inducibility under *vpr*-inducing and -repressing conditions. A 15-kDa protein band was detected with anti-Vpr serum in Vpr-induced cells (Fig. 1A, lane 2). No specific bands were detected in either *vpr*-repressed cells (Fig. 1A, lane 1) or cells that did not contain a *vpr* gene (data not shown).

Vpr-induced small-colony formation and color changes. On the thiamine-containing agar plates (*vpr* gene is repressed), the colonies bearing pREP1N-*vpr* appeared to be normal, i.e., large and creamy white in color. In contrast, when the *vpr* gene was expressed in a thiamine-free environment, *S. pombe* pREP1N-*vpr*-containing colonies appeared to be much smaller and the pigmentation of the colonies changed from creamy white to brown (Fig. 1B). The HIV-1 Vpr-induced change in colony morphology presented after repeated transfers on agar plates under *vpr*-inducing conditions. However, normal colony morphology was restored when the small colonies were transferred to *vpr*-repressing conditions (data not shown).

Vpr-induced morphological changes. Normal cell morphology of *S. pombe* was observed when *vpr* gene expression was repressed on agar plates and in growth medium (Fig. 2A). In contrast, we observed profound morphological changes when the *vpr* gene was expressed. These Vpr-induced morphological changes were highly polymorphic, including elongated (Fig. 2B), spherical (Fig. 2C), irregular (Fig. 2D), and balloon-like cells (Fig. 2E). Vpr-induced morphologic changes started to appear after approximately 24 h of *vpr* inducement and increased proportionally over time. The large majority of cells (>90%) containing pREP1N-*vpr* deviated from their normal morphology after 48 h of incubation.

Prevention of cell proliferation. Time course experiments were conducted to measure the effect of *vpr* gene expression on the cellular proliferation of *S. pombe*. Growth rates of *S. pombe* cells containing pREP1N-*vpr* were compared with those of cells containing the pREP1N vector control. Both types of cells were grown in plasmid-selective EMM with or without thia-

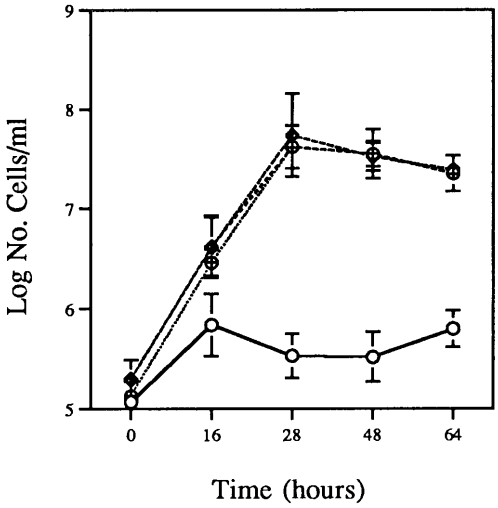

FIG. 3. Effect of HIV-1 Vpr on *S. pombe* cell proliferation. Cells containing pREP1N-*vpr* and pREP1N vectors were grown under gene-repressing or gene-inducing conditions in minimal selection EMM. pREP1N-*vpr*-containing cells grown under gene-repressing and -inducing conditions, circles with crosses and open circles, respectively; pREP1N-containing cells grown under gene-repressing and -inducing conditions, diamonds with the left and right halves shaded, respectively. Error bars, standard errors of the means.

mine. All four cultures were initiated at early log phase (actively growing stage) with an initial concentration of approximately 5.0×10^5 cells per ml. An aliquot of each culture was collected at different times and plated onto YEA agar plates for quantitation. Similar growth dynamics among the *vpr*-repressed *S. pombe* cells and those control cells grown under gene-inducing and -repressing conditions were observed (Fig. 3). These three cultures reached stationary phases after about 28 h, suggesting normal cellular growth of *S. pombe*, with a doubling time of about 4 h. In contrast, the Vpr-induced cells displayed dramatic growth delay. Differences in cell growth rates as great as 2.5 log units between the Vpr-induced and Vpr-repressed cells were observed (Fig. 3).

Vpr arrested *S. pombe* cells in the G_2 phase of the cell cycle. The ability of HIV-1 Vpr to induce cell cycle arrest in *S. pombe* cells was assessed by flow cytometry analysis. In standard EMM, about 70% of *S. pombe* cells normally reside in the G_2 phase of the cell cycle (26). In order to test potential cell cycle G_2 or G_1 arrest, *S. pombe* cells were synchronized by growth in low nitrogen medium for accumulation of G_1 cells or in low glucose medium for accumulation of G_2 cells (7). If Vpr induced G_2 arrest, we would expect a shift of the predominantly G_1 cell population to G_2. Similarly, Vpr-induced G_1 arrest should be represented by a shift of the predominantly G_2 cell population to G_1. As results, expression of HIV-1 *vpr* arrested *S. pombe* cells in the G_2 (Fig. 4A) but not in the G_1 phase of the cell cycle (data not shown). In the G_2-enriched cell population, G_2-phase cells remained unchanged in both Vpr-induced and Vpr-repressed cell cultures even after 72 h of incubation (data not shown). Conversely, the cells started to switch from G_1 to G_2 phase as early as 24 h postinduction. By 48 h, the majority of the cell population rested in the G_2 phase (Fig. 4A). This G_1-to-G_2 shift was not observed under the *vpr*-repressing condition (data not shown). As an additional control, the pREP1N vector was also used in this test, and no G_2 arrest was observed under either gene-inducing or gene-repressing conditions (data not shown).

A conventional septation index method was used as an al-

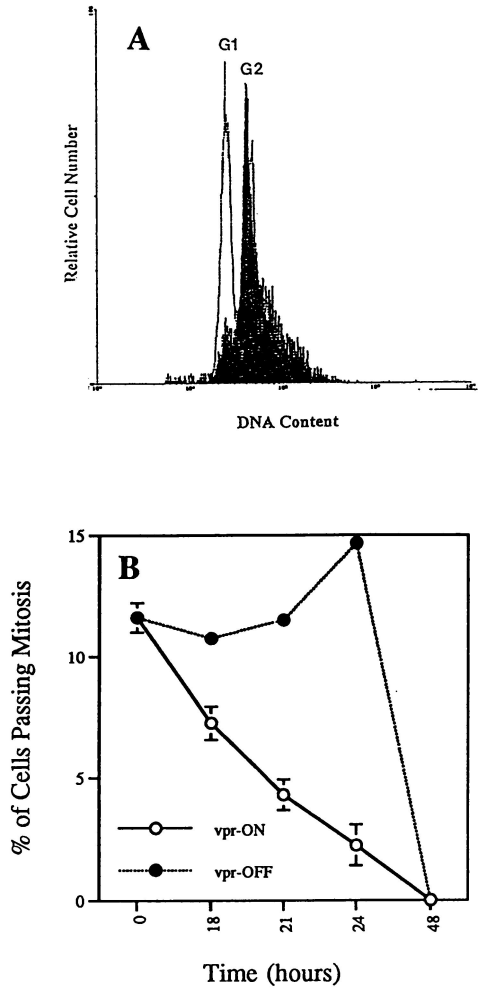

FIG. 4. Vpr-induced cell cycle G_2 arrest. (A) Flow cytometry analysis. The abscissa represents DNA content as measured by the relative scale of propidium iodine fluorescence, indicating the G_1 or G_2 phase of the cell cycle. The ordinate shows the relative number of cells counted. The Vpr-induced cell population is indicated by the closed histogram; the Vpr-repressed cell population is indicated by the open histogram. (B) Septation index analysis. The septation index is represented by the percentage of cells passing mitosis. Error bars, standard errors of the means. (C) Immunoblot of $p34^{cdc2}$ detected by anti-Cdc2 monoclonal antibody. Protein samples were collected at the onset of Vpr induction (*vpr* off) (lane 1) and 42 h after Vpr induction (*vpr* on) (lane 2). The inactive form of $p34^{cdc2}$ is phosphorylated, and the active form is dephosphorylated (8).

ternative means to confirm Vpr-induced G_2 arrest. This method determines the fraction of cells within a population that have a septum, which is indicative of mitotic cell division. In a normal, actively growing cell population, approximately 10 to 20% of cells contain septa (26). Cell arrest is normally characterized by a rapid decrease of septum formation in a population compared with that in actively growing controls. We started with an actively growing cell culture (average of 12% septa) in a concentration of approximately 5×10^5 cells per ml and measured the septation index over time. The *vpr*-repressed culture maintained a normal septation index (12 to 15%) (Fig. 4B) during the actively growing phase, until 48 h, when it reached stationary phase and stopped dividing. In contrast, a rapid decrease of septum formation was found in Vpr-induced cells (Fig. 4B). After 48 h, 0% septa were observed, suggesting that the cells were arrested.

Correlation of Vpr-induced phenotypic changes with increase of phosphorylated p34^{cdc2}. Phosphorylation status of the p34^{cdc2} protein kinase under *vpr*-on and *vpr*-off conditions was measured with an anti-Cdc2 monoclonal antibody by immunoblot analysis. Two closely spaced bands of approximately 34 kDa reacted to the anti-Cdc2 monoclonal antibody under *vpr*-repressing conditions (Fig. 4C, lane 1). The upper band has been shown to correspond to the phosphorylated (inactive) p34^{cdc2} forms, and the lower band represents the dephosphorylated (active) forms (8). Both forms appeared to be approximately equal prior to *vpr* induction (Fig. 4C, lane 1). However, expression of *vpr* resulted in a significant increase in the ratio of phosphorylated to dephosphorylated p34^{cdc2} over time. This increase was more pronounced 42 h after the *vpr* gene was switched on, when the majority of the p34^{cdc2} kinase was in the inactive, phosphorylated form (Fig. 4C, lane 2).

OA relieves Vpr-induced cell cycle block and morphological changes. Treatment of human cells expressing *vpr* with okadaic acid (OA), an inhibitor of protein phosphatase type 2A (PP2A) and PP1, suppressed Vpr-induced cell cycle block (34). We treated the Vpr-induced cells with OA to test whether *S. pombe* protein phosphatases also play a role in the Vpr-induced cell cycle block. The septation index was used as a marker to monitor cell cycle progression. Similar to what was shown earlier (Fig. 4B), Vpr-repressed cells divided normally, with an average of 15% of cells passing through mitosis. Vpr-induced cells showed a continuous decrease in septum formation, and the large majority of the cells (>98%) stopped dividing at 22 h post-*vpr*-inducement, suggesting cell cycle arrest. In contrast, both Vpr-induced and Vpr-repressed cells showed similar septation profiles and active division when treated with OA (Fig. 5), suggesting that Vpr-induced cell cycle block was suppressed by OA. Treatment with OA also delayed Vpr-induced morphologic changes. Vpr-induced cells normally start to show aberrant morphologies at approximately 24 h post-*vpr* inducement; no apparent morphological changes were detected at the same time in OA-treated cells. A limited extent of morphological changes appeared after 40 h of *vpr* induction (data not shown). As an additional control, both Vpr-induced and -repressed cells were also treated with methyl OA, which is structurally similar to OA but lacks phosphatase-inhibitory activity (12). This compound showed no effect on either the Vpr-induced cell cycle arrest or morphological changes (data not shown).

Cell cycle G_2 arrest was independent of cell size and morphological changes. We measured cell cycle distribution of the cells as categorized by the forward-angle light scatter signal (correlate of cell size). We gated the *S. pombe* cells into two subpopulations based on light scatter (low versus high forward angle) (Fig. 6A). The majority of the large-cell population (Fig. 6B [black area]) were in the G_2 phase of the cell cycle, whereas the small-cell population (Fig. 6B [open area]) contained cells from the G_1 to G_2 phases of the cell cycle. When measured as one population (Fig. 6B), most (>90%) of the cells were in G_2 phase of the cell cycle.

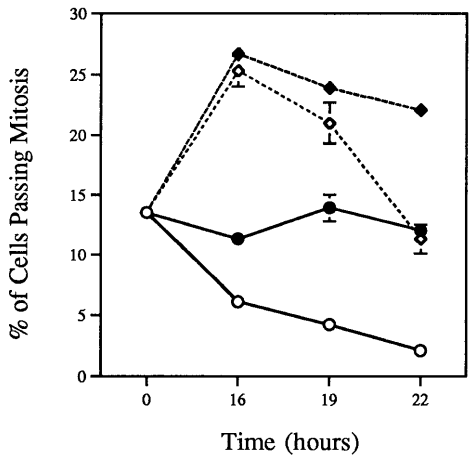

FIG. 5. Suppression of Vpr-induced cell cycle arrest by OA as measured by septation index analysis. The septation index is represented by the percentage of cells passing mitosis. OA was used at 10 μM in OA-treated cells (diamonds). Circles show data for cells grown without OA. Open and closed symbols show data for cells grown under gene-inducing and -repressing conditions, respectively. Error bars, standard errors of the means.

DISCUSSION

We have successfully expressed the HIV-1 *vpr* gene in *S. pombe* cells. By using an inducible *nmt1* promoter, we were able to induce and repress HIV-1 *vpr* gene expression to demonstrate Vpr-specific effects on cellular functions. We observed that the effects of Vpr in *S. pombe* cells are similar to those which have been described for human cells. Specifically, Vpr alters cell morphology, prevents cell proliferation, and induces the arrest of cells in the G_2 phase of the cell cycle. In addition, we also observed that Vpr induces small-colony formation and pigmentation change.

The specific induction of the G_2 arrest by Vpr was supported by the observations that only G_2 arrest and no G_1 arrest was observed (Fig. 4A), and the G_2 arrest was not detected when the *vpr* gene was repressed or when a vector control was used. This cell cycle-arresting effect was further confirmed by a septation index analysis. Using this method, we observed that the induction of *vpr* gene expression resulted in a quick decrease in septum formation, with no septa observed 48 h after *vpr* inducement (Fig. 4B).

Consistent with the recent reports that Vpr induces G_2 arrest by preventing the activation of p34^{cdc2} (9, 15, 34), we observed by immunoblot analyses an increase in the phosphorylated form of p34^{cdc2} (the inactive form), which correlated with Vpr-induced G_2 arrest (Fig. 4C). It is noteworthy that the p34^{cdc2} human homologs have been identified in a variety of organisms, including *S. pombe* (19), mouse (36), *Drosophila* (14), *Xenopus* (25, 33), maize (*Zea mays*) (5), goldfish (11), and *Entamoeba histolytica* (21) cells, suggesting that the effect of HIV-1 Vpr on cell cycle G_2 arrest may be mediated through a highly conserved cellular process.

Re et al. (34) reported that the protein phosphatase inhibitor OA could release the cell cycle block caused by Vpr in human cells, suggesting the involvement of protein phosphata-

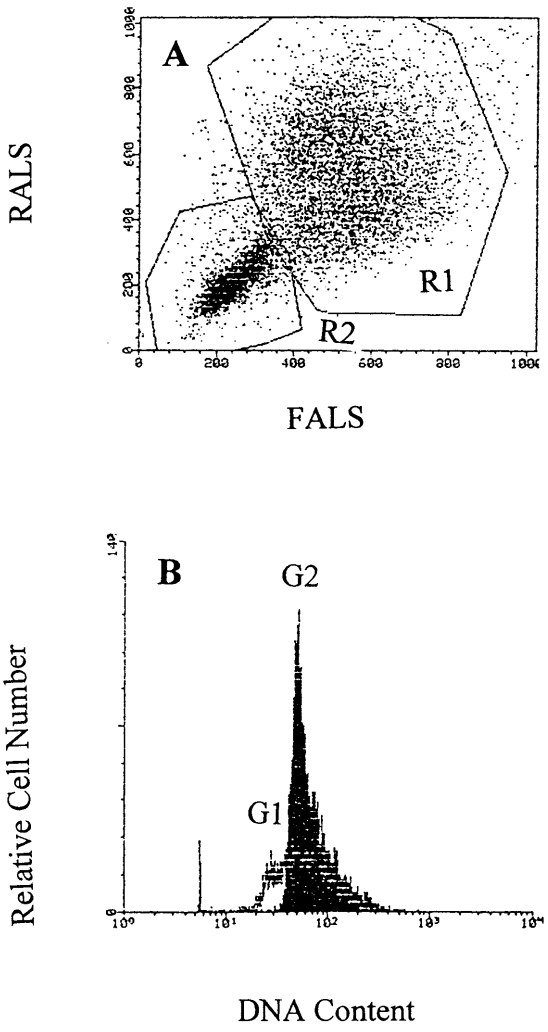

FIG. 6. (A) Correlation of G_2 cell cycle arrest with cellular light scatter properties. (A) Light scatter properties of Vpr-induced *S. pombe* cells represented as a dot plot of right-angle light scatter (RALS) (correlate of intracellular complexity) on the *y* axis vs. forward-angle light scatter (FALS) (correlate of cell size) on the *x* axis. (B) Electronic analysis regions were drawn on the larger cells (R1) and the small cells (R2). The DNA content (FL2 fluorescence) of each region is represented (R1, open histogram; R2, closed histogram).

ses in cell cycling. We observed that treatment of Vpr-induced cells with OA not only suppressed cell cycle arrest but also blocked morphological changes (Fig. 5). This result suggests that *S. pombe* protein phosphatases may also be involved in Vpr-induced morphological changes in addition to cell cycling. Interestingly, the suppressive effect of OA on Vpr-induced morphological changes appears to be a transient event, as some cells eventually began to change their morphology but to a lesser extent (data not shown). Although the septation index also showed an initial decline 20 h post-*vpr* inducement (Fig. 5), cells continued to divide during the 40-h testing period (data not shown), suggesting that OA was more efficient in suppressing Vpr-induced cell cycle arrest than in suppressing morphological changes. At present, we have no additional evidence to elucidate the specific mechanisms mediating these cellular effects. We speculate that the Vpr-induced G_2 arrest and morphological changes are affected by different protein phosphatases and controlled by two separate functions of Vpr. Further tests to confirm the involvement of protein phosphatases in Vpr-induced cellular changes are warranted. A more definitive answer could potentially be obtained by expressing *vpr* in various genetic mutants that are defective in protein phosphatase production. OA is an effective inhibitor of *S. pombe* PP2A and also has less extensive inhibitory effect on PP1 (18). Notably, deletion of the *ppa2* gene, encoding PP2A, revealed the same protein phosphorylation pattern as that for OA, suggesting that *ppa2* is the genetic locus controlling OA sensitivity (18). As PP2A is known to negatively regulate mitosis in *S. pombe* (17), we anticipate that Vpr-induced cell cycle G_2 arrest and other cellular changes could be potentially linked to interaction of Vpr with PP2A and other protein phosphatases in *S. pombe*. Studies designed to test this hypothesis are currently in progress.

S. pombe G_2-arrested cells, induced by either exogenous (e.g., radiation) or endogenous (e.g., *cdc* mutation) factors, typically become uniformly elongated. This is markedly contrasted by the highly diversified cell length and morphology observed in Vpr-induced G_2 cells. Comparison of the two cell populations as categorized by forward-angle light scatter (correlate of cell size) showed that G_2 cells were distributed by both the large- and small-cell populations (Fig. 6), suggesting that Vpr-induced cell cycle G_2 arrest is independent of cell size and morphological changes. At present, the molecular mechanisms by which Vpr affects cell morphology is unknown. Since profound cellular structural changes were observed in Vpr-induced cells, we hypothesize that Vpr could affect cell morphology by disturbing the cytoskeletal structure. A number of tests have been designed in our laboratory to test this hypothesis. We observed that Vpr induces small-colony formation, which probably coincides with the cellular growth arrest induced by Vpr. The mechanism of Vpr-induced color change of colonies in *S. pombe* is unknown. A previous study showed that *S. pombe* colonies mutated at an *ade6* locus and grown in an adenine-free environment changed to a red color because of the accumulation of an adenine intermediate that polymerized to form a red pigment (28). It is possible that a metabolic process altered by Vpr caused an accumulation of an as yet unknown brownish pigment.

In summary, our studies suggest that HIV-1 Vpr-induced cellular changes in *S. pombe* mimic those found in human cells. In addition, our results further suggest that Vpr-induced G_2 arrest and cell morphological changes may be controlled by two different functions of Vpr. Since *S. pombe* is a relatively simple monocellular eukaryote and a number of cell cycle control genes are interchangeable between human and fission yeast cells (13, 19, 29, 32), *S. pombe* provides a useful model system to study genetic and biochemical aspects of HIV-1 *vpr* gene functions.

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