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Suppression of v-*src* Transformation by the *drs* Gene

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Previously, we isolated a novel gene, *drs*, which was downregulated by retroviral oncogenes such as v-*src* and v-K-*ras*, from a cDNA library of primary rat embryo fibroblasts. Experiments using a temperature-sensitive mutant of the v-*src* gene indicated that downregulation of *drs* mRNA was dependent on functional expression of v-Src. In addition, expression of *drs* mRNA was also reduced by serum stimulation of G_0-arrested normal rat fibroblast cells. To clarify the function of the *drs* gene in cell transformation and proliferation, we introduced *drs* linked to a potent promoter into a normal rat cell line, F2408, and examined the effect of ectopic expression of exogenous *drs* on the transformation by the v-*src* gene and growth properties. Cells expressing exogenous *drs* gene showed significantly decreased efficiency of transformation by v-*src* irrespective of functional expression of v-Src kinase, while the growth rate and G_1/S progression of the cells were not suppressed by expression of exogenous *drs* gene, indicating that *drs* has the ability to suppress v-*src* transformation without disturbing cell proliferation.

Transformation by viral oncogenes induces a variety of cellular changes such as cell rounding, loss of contact inhibition, decrease of serum requirement for cell proliferation, and anchorage-independent growth. The v-*src* oncogene of Rous sarcoma virus (RSV) has been most intensively investigated (29). The product of v-*src* is a membrane-associated phosphoprotein, $pp60^{v\text{-}src}$, which has tyrosine-specific protein kinase activity (9, 20, 32). The v-*src* gene also positively or negatively alters the expression of many cellular genes (1, 6, 12, 14, 16, 28, 33, 40, 44, 45, 49). A few of the genes whose expression is negatively regulated by v-*src* have been shown to function as tumor suppressor genes (11, 33, 41). We have reported that a suppressive factor(s) for v-*src* transformation is expressed in primary rat embryo fibroblasts (REF) (25, 51, 52). On searching for such transformation suppressor genes, we recently isolated a novel gene, *drs* (downregulated by v-*src*), which was expressed in normal rat fibroblast cells but completely suppressed in the cells transformed by the v-*src* gene, from a cDNA library of REF (42). The *drs* gene was also demonstrated to be downregulated by other retroviral oncogenes such as v-*fps*, v-*ras*, v-*mos*, v-*sis*, and v-*abl*. The molecularly cloned cDNA of *drs* was about 1.8 kb in size, containing an open reading frame composed of 464 amino acid residues. This protein had one transmembrane domain in the C terminus and three consensus repeats conserved in various numbers in the extracellular domain among the selectin family of adhesion molecules and complement binding proteins (4, 30). Marked downregulation of *drs* mRNA in oncogene-transformed cells suggests that the *drs* gene plays a role in suppression of transformation.

To clarify the function of *drs* for cell transformation, we initially investigated the correlation between downregulation of *drs* mRNA and functional expression of the v-*src* gene. To perform this experiment, we used a rat F2408 cell clone (OS7-2) containing a temperature-sensitive mutant (OS122) of RSV (13, 38, 39, 54). As shown in Fig. 1, OS7-2 displays round refractile morphology typical of transformed cells when incubated at 35°C. When the temperature was shifted to 39°C from 35°C, the morphology of OS7-2 was gradually converted to a flat phenotype within 24 h. Tyrosine kinase activity of v-Src by in vitro kinase assay with anti-Src serum was also reduced by temperature shift from 35 to 39°C (Fig. 2A). The change of tyrosine kinase activity roughly paralleled the morphological change in OS7-2 cells. The level of *drs* mRNA gradually increased after the temperature increase to 39°C in parallel with the decrease of the kinase activity in OS7-2 cells (Fig. 2B). In the F2408 cell clone S7-1 (23), containing wild-type RSV, the transformed morphology, tyrosine kinase activity, and level of *drs* mRNA were not changed by temperature shift (Fig. 1 and 2). The expression of *drs* mRNA in untransformed F2408 cells was also not affected by temperature shift (Fig. 2B). These results indicate that downregulation of *drs* mRNA depends on functional expression of v-Src tyrosine kinase and parallels the expression of transformed morphology.

To examine whether *drs* mRNA expression varies with cell cycle progression, F2408 cells arrested in G_0 phase by serum starvation were stimulated by serum (10% fetal calf serum [FCS]) and the change in the amount of *drs* mRNA was examined (Fig. 3). Under these conditions, G_0-arrested F2408 cells synchronously progressed to S phase 12 h after serum stimulation (26). Expression of *drs* mRNA was gradually reduced until 5 h after serum stimulation (Fig. 3). The level of *drs* mRNA was unchanged from 5 to 18 h (the peak of S phase) (Fig. 3). These results suggest that expression of the *drs* gene is also regulated during the cell cycle.

To assess the function of the *drs* gene in v-*src* transformation and cell proliferation, we constructed a recombinant plasmid, pSRαNeo/drs, that expresses the *drs* gene under the potent promoter-enhancer SRα (50), and transfected this plasmid into an F2408 cell line. After G418 selection, six independent G418-resistant clones (F-drs-2, -3, -4, -5, -7, and -10) were isolated, and expression of exogenous *drs* gene in these clones was examined by Northern blot hybridization. As shown in Fig. 4A, three clones (F-drs-2, -4, and -7) expressed high levels of exogenous *drs* mRNA (upper band) in addition to expressing endogenous *drs* mRNA (lower band), while another three clones (F-drs-3, -5, and -10) expressed only endogenous *drs* mRNA. The cell morphologies of F2408 and the six G418-

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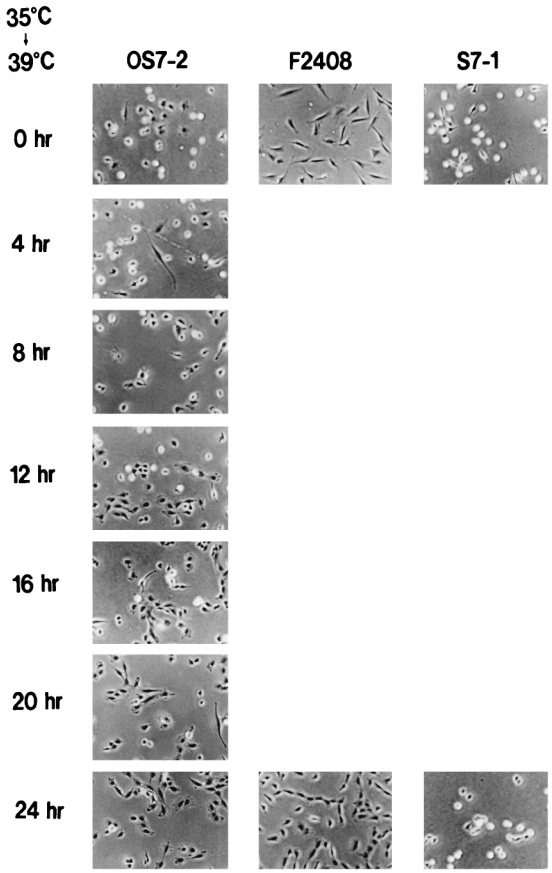

FIG. 1. Morphological change of OS7-2 cells by temperature shifting. F2408 is a rat fibroblast cell line established from REF of Fisher rat (13). S7-1 is an F2408 cell line transformed by the SR-D strain of RSV (23). OS7-2 is an F2408 cell line containing a temperature-sensitive mutant (OS122) of RSV (38, 39, 54). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% FCS (growth medium). OS7-2, S7-1, and F2408 cells were inoculated into plastic dishes containing growth medium and incubated at 35°C (permissive temperature). After incubation for 24 h, the culture temperature was increased to 39°C. At 0, 4, 8, 12, 16, 20, and 24 h after temperature shifting (0 and 24 h for S7-1 and F2408), cell morphologies were observed with a phase-contrast microscope and photographed.

resistant clones were similar (data not shown). To clarify whether the *drs* gene has the activity to suppress v-*src* transformation, F2408 and these clones were infected with a high titer of a recombinant murine retrovirus (MRSV) containing the v-*src* gene (2), and the colony-forming abilities in soft agar and the focus-forming abilities in liquid culture of these cells were investigated. As shown in Fig. 5A, the colony-forming efficiencies of the clones expressing exogenous *drs* (F-drs-2, -4, and -7) were significantly lower than those of F2408 and the clones expressing only endogenous *drs*. Focus-forming efficiencies of F-drs-2, F-drs-4, and F-drs-7 by MRSV were also markedly decreased compared with those of F2408 and F-drs-10 (Fig. 5B). These results suggest that the *drs* gene acts to suppress v-*src* transformation. To exclude the possibility that expression of functional v-Src protein was inhibited in the clones expressing exogenous *drs* gene, the tyrosine kinase activity of v-Src protein in MRSV-infected cells was examined by in vitro protein kinase assay with anti-Src serum. Figure 6 shows the results. v-Src kinase activities of F-drs-2, -4, and -7 infected with MRSV were not reduced compared with those of MRSV-infected F2408, F-drs-3, -5, and -10, indicating that suppression of v-*src* transformation in the clones expressing exogenous *drs* gene is not due to the reduced expression of v-Src tyrosine kinase. We also examined the expression of exogenous and endogenous *drs* mRNA in mock- and MRSV-infected F-drs-7 cells. As shown in Fig. 4B, the level of endogenous *drs* mRNA was reduced by MRSV infection, whereas the expression of exogenous *drs* mRNA driven from the SRα promoter was not affected by MRSV, confirming that v-*src* certainly functions to downregulate endogenous *drs* in MRSV-infected F2408 cells expressing exogenous *drs*. However, the *drs* gene driven from the exogenous promoter was not downregulated by v-*src*. This

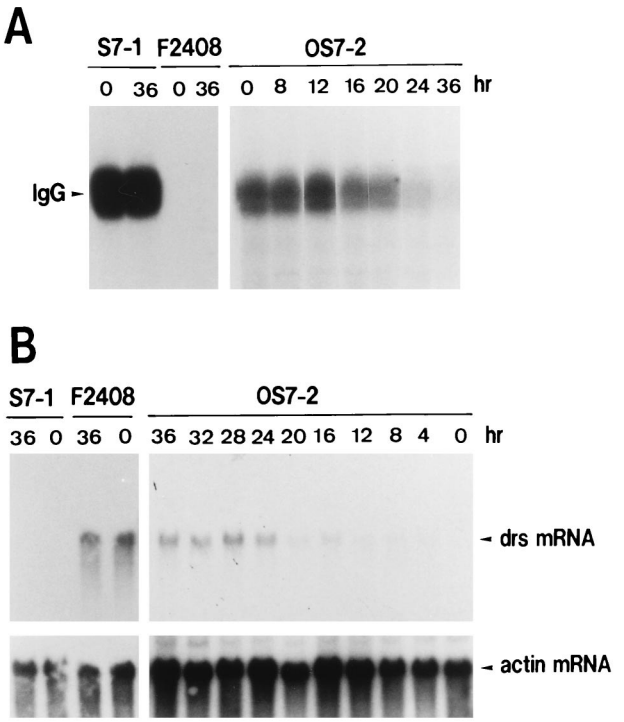

FIG. 2. Alterations of protein kinase activity of v-Src (A) and expression of *drs* mRNA (B) by temperature shifting in OS7-2 cells. (A) In vitro protein kinase assay. Temperature shifting was performed as described in the legend to Fig. 1. For examination of protein kinase activity of v-Src, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 20 mM Tris-HCl [pH 7.4], 150 mM sodium chloride, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 20 μg of aprotinin/ml) and centrifuged at 13,000 × *g* for 30 min at 4°C. The resulting supernatant was used for kinase assay of v-Src as described by Inoue et al. (22). Cell extracts containing 100 μg of protein were immunoprecipitated with 5 μl of anti-Src serum for 1 h at 4°C. The immunocomplexes were bound to protein A-Sepharose, washed three times with RIPA buffer, and suspended in 20 μl of kinase buffer (20 mM Tris-HCl [pH 7.4], 10 mM $MnCl_2$) containing 5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham). After incubation for 10 min at 20°C, the reaction was stopped by addition of 15 μl of 4× Laemmli-SDS buffer (0.25 M Tris-Cl [pH 6.8], 40% glycerol, 8% SDS, 20% 2-mercaptoethanol, 0.01% bromophenol blue) and boiled for 2 min. The reaction mixtures were centrifuged at 10,000 × *g* for 2 min, and samples of the supernatant were analyzed by SDS-polyacrylamide gel electrophoresis. The position of the phosphorylated immunoglobulin G (IgG) heavy chain is indicated. (B) Northern blot analysis. Cellular RNA was isolated from cells by the guanidium isothiocyanate-cesium chloride method (8). Samples (20 μg) of total RNA from the cells were subjected to electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde and transferred to a nylon filter. The filter was hybridized at 42°C overnight in a solution containing 50% formamide, 0.6 M sodium chloride, 60 mM sodium citrate, 0.2% SDS, 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 50 μg of herring sperm DNA/ml with a labeled DNA probe. The hybridized filter was washed with 15 mM sodium chloride–1.5 mM sodium citrate–0.1% SDS at 50°C and autoradiographed. Probes used in this experiment were a *drs* DNA fragment and a human β-actin DNA fragment (37).

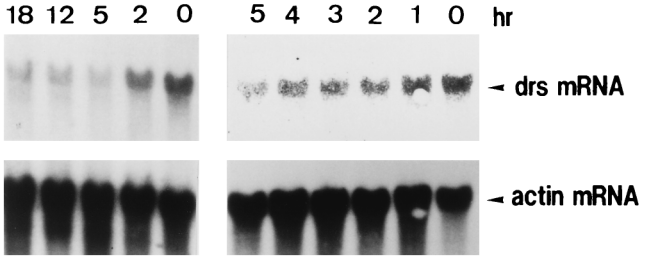

FIG. 3. Expression of *drs* mRNA during cell cycle progression of F2408 cells. Confluent cultures of F2408 cells were serum starved for 24 h and then supplemented with 10% FCS. At 0, 1, 2, 3, 4, 5, 12, and 18 h after serum stimulation, cellular RNA was isolated. Isolation of cellular RNA and Northern blot hybridization were carried out as described in the legend to Fig. 2B.

result further supports our conclusion that an ectopically expressed exogenous *drs* acts to suppress transformation by v-*src*.

To further confirm the correlation between expression of exogenous *drs* and suppression of transformation by v-*src*, we constructed a recombinant retrovirus containing the *drs* gene and the puromycin-resistant gene as a selective marker (pBabePuro-drs) and infected F2408 cells with the virus. After incubation in selection medium containing 1 μg of puromycin/ml, puromycin-resistant cells were pooled, infected with MRSV, and inoculated into soft agar. F2408 cells infected with pBabePuro-drs virus expressed a considerable amount of viral mRNA hybridized with *drs* cDNA (Fig. 4C). As shown in Fig. 5C, the colony-forming efficiency of F2408 cells containing pBabePuro-drs virus (F/pBP-drs) by MRSV was markedly decreased compared with that of F2408 cells containing vector virus (F/pBP). v-Src kinase activities in MRSV-infected F/pBP and F/pBP-drs cells were also almost similar (Fig. 6), confirming the suppression function of *drs* for v-*src* transformation. In addition, we also examined colony formation in soft agar and focus formation of F2408, F-drs-2, F-drs-4, F-drs-7, and F-drs-10 cells by a murine retrovirus containing v-K-*ras* (Ki-MSV). As shown in Fig. 5D (soft agar assay) and E (focus assay), transformation efficiencies of F-drs-2, F-drs-4 and F-drs-7 were also significantly decreased compared with those of F2408 and F-drs-10. These results, together with those of v-*src* transformation, indicate that ectopic expression of the *drs* gene suppresses transformation by viral oncogenes such as v-*src* and v-K-*ras*.

To investigate the effect of ectopic expression of the *drs* gene on growth properties of F2408 cells, we examined the growth rates of F2408, F-drs-2, F-drs-7, and F-drs-10 cells. As shown in Fig. 7A, expression of exogenous *drs* gene did not affect the growth rate of the cells. The growth rates of F/pBP and F/pBP-drs cells were also similar (data not shown). To examine whether overexpression of exogenous *drs* gene suppresses G_1/S progression of the cell cycle, F2408, F-drs-2, F-drs-7, and F-drs-10 cells were arrested in G_0 phase by serum starvation and stimulated with serum. Progression of the cell cycle of these cells was examined by flow cytometry-activated cell sorting (Fig. 7B). The entry into S phase at 18 h after serum stimulation was not reduced by expression of exogenous *drs* gene in F-drs-2 and F-drs-7 cells compared with that of F2408 and F-drs-10 cells, indicating that the exogenous *drs* gene does not suppress G_1/S progression of the cell cycle. From these results, we concluded that the *drs* gene acts to suppress transformation induced by v-*src* and v-*ras* without affecting cell proliferation in F2408 cells.

Previously, we showed that *drs* mRNA was markedly reduced in rat cell lines transformed by v-*src*, v-*fps*, v-*ras*, v-*mos*, v-*sis*, v-*abl*, or middle T antigen of polyomavirus but not in cell lines transformed by large T antigen of simian virus 40 or the E6 and E7 genes of human papillomavirus type 16 (24, 42). As the oncogenes that reduced *drs* mRNA are considered to act upstream of the p42/p44 mitogen-activated protein (MAP) kinase pathway (19, 43), we speculated that expression of the *drs* gene is negatively regulated by mitogenic signals from growth factors downstream of the MAP kinase pathway but upstream of the cyclin/CDK-Rb pathway. In fact, as shown in Fig. 3, the level of *drs* mRNA was considerably reduced by serum stimulation of G_0-arrested cells although the expression was not completely suppressed. This result suggested that downregulation of *drs* mRNA by mitogenic signals plays a role in the progression of the cell cycle. However, overexpression of the exogenous *drs* gene by the SRα promoter did not affect cell proliferation (Fig. 7). Although mitogenic factors in the serum act to modestly regulate the expression of *drs* gene, the downregulation might not be critical for G_1/S progression of the cell

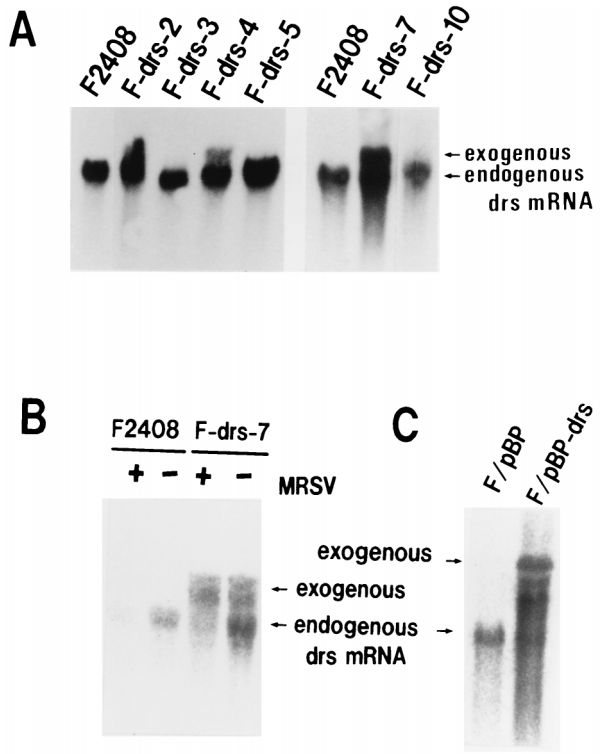

FIG. 4. Expression of endogenous and exogenous *drs* mRNA in F2408 and F-drs cells (A and C) and the effect of MRSV infection on *drs* mRNA expression (B). (A) The expression vector in this experiment was pSRαNeo, which contains the SRα promoter (50) for efficient expression of inserted cDNA and the neomycin-resistant gene as a selective marker. A 1.8-kb cDNA fragment (*Bam*HI) containing the open reading frame of the *drs* gene was inserted into the *Bam*HI-cleaved pSRαNeo vector. The recombinant plasmid, pSRαNeo/drs, in which a *drs* gene was inserted in the sense orientation, was used for DNA transfection experiments. pSRαNeo/drs plasmid DNA was introduced into F2408 by the calcium-phosphate transfection method (17), and G418-resistant clones were isolated. Isolation of cellular RNA and Northern blot hybridization were carried out as described in the legend to Fig. 2B. (B) Cellular RNA was isolated 3 days after MRSV infection. The upper and lower bands indicate exogenous and endogenous *drs* transcripts, respectively. (C) A 1.8-kb *drs* cDNA fragment was inserted into *Bam*HI-cleaved pBabePuro retrovirus vector (36) in the sense orientation. The recombinant plasmid, pBabePuro-drs, was introduced into a packaging cell line, ψ2 (34), by DNA transfection, and puromycin-resistant clones containing the *drs* gene were isolated. F2408 cells were infected with culture medium of cells containing pBabePuro-drs or vector (pBabePuro) virus, and puromycin-resistant cells were pooled (F/pBP-drs and F/pBP).

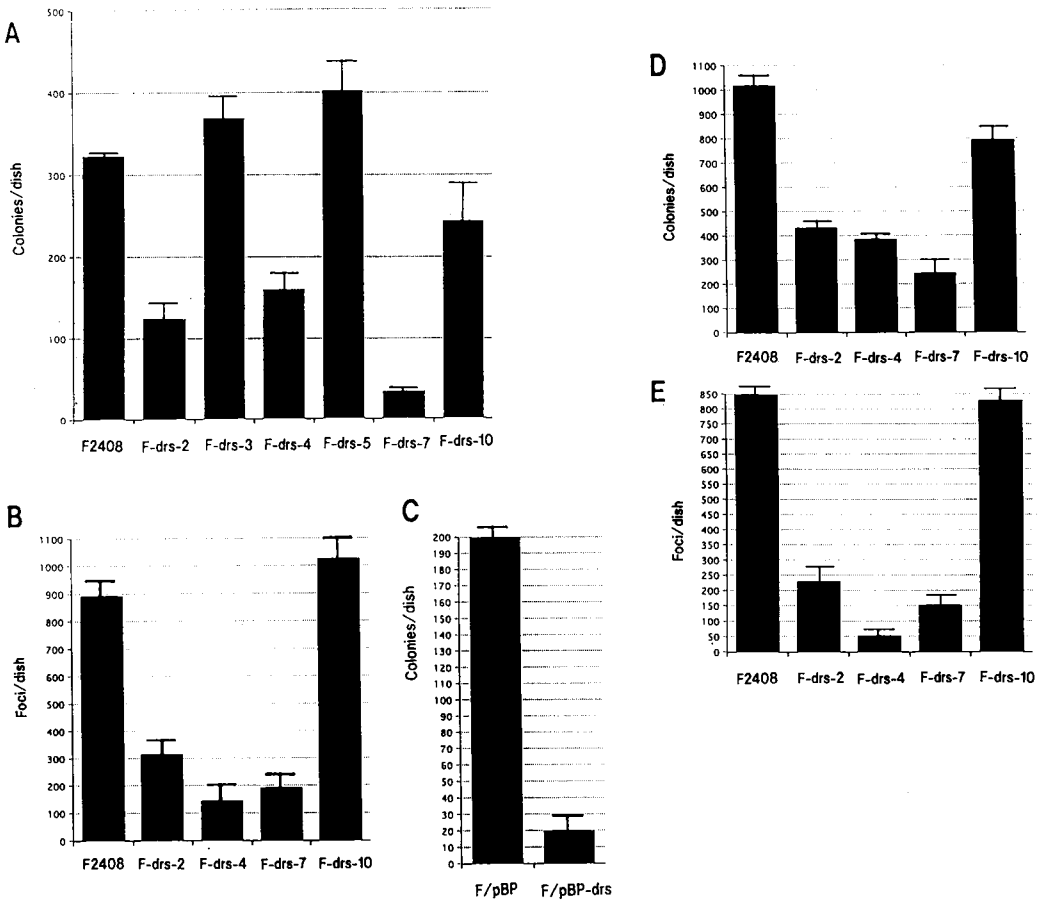

FIG. 5. Transformation of F2408 and F-drs cells by MRSV and Ki-MSV. F2408 and F-drs clones transfected with pSRαNeo/drs plasmid were infected with MRSV (A and B) or Ki-MSV (D and E). F/pBP and F/pBP-drs cells were infected with MRSV (C). For virus infection, inocula of 2×10^5 cells were plated in 60-mm-diameter plastic dishes with growth medium and incubated overnight at 37°C. After treatment of the cultures with Polybrene (2 μg/ml) for 30 min at 37°C, the medium was removed and 0.3 ml of virus preparation was added to each culture. After adsorption for 1 h at 37°C, the cultures were covered with growth medium and incubated at 37°C. For soft agar assays (A, C, and D), 3 days after virus infection, the cells were trypsinized and portions of 10^4 cells were inoculated into 0.4% soft agar. Colonies were scored after incubation for 2 weeks. For focus assays (B and E), transformed foci were scored 10 days after virus infection.

cycle. However, we cannot completely rule out the possibility that the level of exogenous *drs* mRNA is not sufficient to affect cell proliferation in this cell line.

Figure 4B shows that introduction of the v-*src* gene reduced the level of endogenous *drs* mRNA but did not affect that of exogenous *drs* mRNA driven by the SRα promoter. This result also implies that downregulation of the *drs* gene by v-*src* is caused by transcriptional repression in the 5′ regulatory region of the *drs* gene. Activated MAP kinase moves into the nucleus and acts to regulate gene transcription (19, 43). Viral oncogenes such as v-*src* and mitogenic factors in serum may repress transcription of the *drs* gene through the MAP kinase pathway. The mechanism of downregulation of the *drs* gene by oncogenes and mitogens still remains to be worked out.

Introduction of the v-*src* gene into normal cells results in multiple cellular events including morphological change, activation of the mitogenic pathway, and anchorage-independent growth. Overexpression of the *drs* gene with the SRα promoter significantly suppressed anchorage-independent growth and focus formation by v-*src* without disturbing usual cell proliferation. Expression of v-Src tyrosine kinase by MRSV infection was not affected by ectopic expression of the *drs* gene (Fig. 6), indicating that *drs* acts to suppress v-*src* transformation after function of v-Src kinase. The most prominent biochemical change induced by v-Src is an extensive tyrosine phosphorylation of cellular proteins (29). Most of these target proteins of v-Src kinase are localized in the focal adhesions linked to the plasma membrane. Deregulated phosphorylation of these focal adhesion proteins, such as paxillin, focal adhesion kinase, talin,

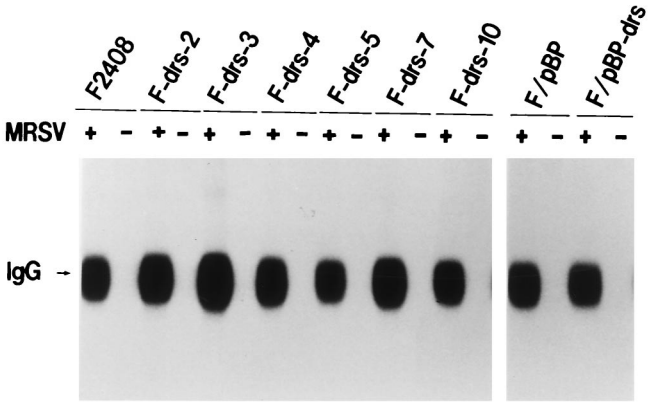

FIG. 6. v-Src kinase activities in MRSV-infected F2408 and F-drs cells. Three days after MRSV infection, the mock- and MRSV-infected cells were lysed in RIPA buffer. The cell lysates containing 100 μg of protein were used for protein kinase assay of v-Src as described in the legend to Fig. 2A.

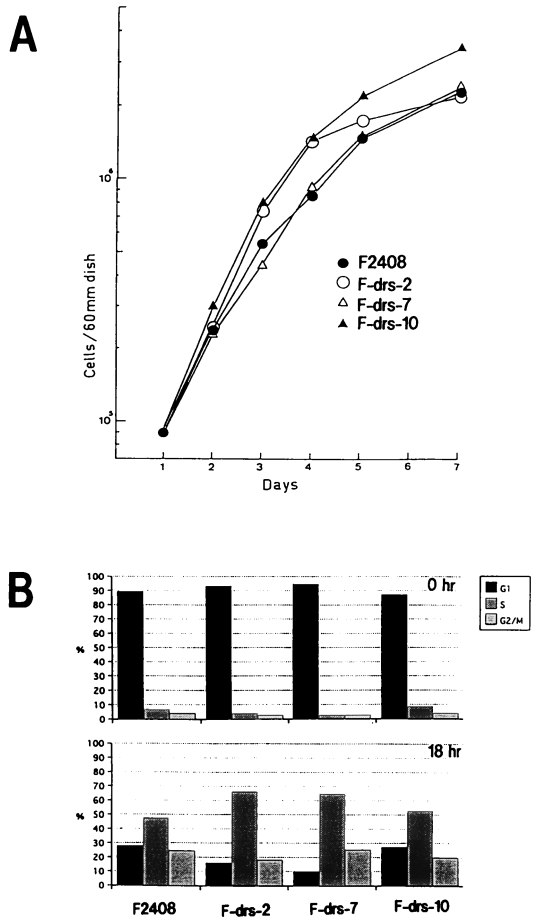

FIG. 7. Growth curves (A) and cell cycle analyses (B) of F2408 and F-drs clones. Cell cycle analysis was carried out by measuring the cellular DNA content by flow cytometry-activated cell sorting as previously described (26). To isolate and stain the cell nuclei, the Cycle TEST PLUS DNA Reagent Kit (Becton Dickinson) was used. Cells were washed with phosphate-buffered saline, suspended in sodium citrate buffer, quickly frozen in a bath of dry ice-methanol, and stored at −80°C until use. The cells were thawed, and their DNA was stained with Cycle TEST reagent by the procedures recommended by the manufacturer. The fluorescence of the cells was measured with a FACScan system (Becton Dickinson), and the percentages of the cells in G_1, S, and G_2/M phases were determined by the CellFit program (Becton Dickinson).

and tensin, is considered to be the cause of rounding and disordered proliferation of the cells. The gene structure of *drs* implies the membrane-associated localization of Drs protein. It seems possible that the product of the *drs* gene interacts with these focal adhesion proteins at the membrane and interferes with transformation by v-Src. The *drs* gene also contains repeated motifs conserved in the extracellular domain among selectin family adhesion molecules (4, 30). Three selectins (L-selectin, E-selectin, and P-selectin) are included in this family. They share a similar molecular structure, consisting of an amino-terminal C-type lectin domain, an epidermal growth factor-like domain, from two to nine short complement regulatory repeats, a transmembrane domain, and a short cytoplasmic tail (5, 27, 31, 48, 53). The complement regulatory repeats of the selectin family had homology with three consensus repeats of the *drs* gene (42). The selectin family is considered to be crucial for the initial step of leukocyte-endothelial interaction in response to inflamatory stimuli such as injury and infection (4, 30). Recently, in addition to adhesion function, E-selectin was shown to associate with actin-associated proteins such as α-actinin, vinculin, filamin, paxillin, and focal adhesion kinase (57). L-selectin has also been reported to act as a signaling molecule which activates MAP kinase and Ras pathways through tyrosine phosphorylation (7, 56). In this regard, selectins resemble another family of adhesion molecules, integrins. The integrin-mediated signaling pathway is thought to play an important role in adhesion-dependent cell cycle progression as well as regulation of the cytoskeleton (3, 21, 46, 47). Accumulating evidence indicates that some of the adhesion molecules localized in the plasma membrane are able to act as tumor suppressor genes (15, 18, 55). The *drs* gene may also function as a receptor for adhesion signaling and be involved in the anchorage-dependent pathway. Further investigation of the *drs* gene is necessary to clarify the mechanism of suppression of transformation. Recently, we found that the *drs* gene is highly homologous (80% in nucleotide sequence) to a human gene which is deleted in patients with X-linked retinitis pigmentosa (10, 35), suggesting that *drs* has significant physiological functions in a variety of cell types.

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