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The Truncated Form of the Epstein-Barr Virus Latent-Infection Membrane Protein Expressed in Virus Replication Does Not Transform Rodent Fibroblasts

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The gene encoding the Epstein-Barr virus membrane protein LMP, expressed in latent infection, is known to induce morphologic changes and some loss of contact inhibition in NIH 3T3 cells as well as profound loss of contact inhibition and of anchorage dependence in Rat-1 cells. Another form of LMP (D1LMP), deleted for the amino terminus and first four putative transmembrane domains of LMP, was recently shown to be expressed late in Epstein-Barr virus replication. We now demonstrate that D1LMP has no transformation-associated phenotypic effect in Rat-1 cells and does not significantly affect LMP-induced Rat-1 cell transformation. LMP activity and D1LMP inactivity in inducing anchorage-independent growth are not restricted to Rat-1 cells, but are also evident in BALB/c 3T3 cells. In both cell types, loss of contact inhibition and anchorage independence are acutely evident after LMP expression. Although newly transfected polyclonal Rat-1 or BALB/c cells have a lower agar cloning efficiency than established LMP-expressing clones, this is attributable, at least in part, to their lower average LMP expression, since among clones of transfected cells, higher cloning efficiencies correlated with higher levels of LMP. LMP is bound to the vimentin cytoskeletal network in rodent fibroblasts as it is in transformed lymphocytes, whereas D1LMP showed no detectable cytoskeletal binding, suggesting that cytoskeletal association may be integral to LMP-mediated cell transformation. LMP association with the cytoskeleton in latently infected, growth-transformed lymphocytes and LMP-transformed rodent fibroblasts, correlated with the lack of both rodent cell-transforming activity and cytoskeletal association of D1LMP supports working hypothesis that cytoskeletal association is important in LMP transforming activity.

Epstein-Barr virus (EBV) is a prevalent human herpesvirus. It infects and persists latently in B lymphocytes, acutely and efficiently causing increased B-lymphocyte proliferation. The proliferating B cells can form tumors in immune-suppressed humans or in marmosets (23). In latently infected lymphocytes, EBV genes associated with virus replication are not expressed, and virus gene expression is limited to several nuclear proteins and a 386-amino-acid integral membrane protein, LMP (12, 15, 31; for review, see reference 11). Since these specific genes are expressed in latent EBV infection, they may be important for maintaining latent infection, for continued cell proliferation, or for tumor induction.

Previous assays of the effects of individual EBV latent-infection genes on rodent fibroblast cell growth revealed that LMP caused transfected Rat-1 cell clones to grow more densely, to lose contact inhibition, to lose anchorage dependence, and to be tumorigenic in nude mice. These data support the hypothesis that LMP plays an important role in EBV-induced lymphocyte transformation. Somewhat against this hypothesis is the observation that unlike the products of other latent infection cycle genes, a form of LMP is also specifically expressed late in virus replication (4, 16, 24, 30). However, this late virus-replication-associated LMP (D1LMP) could be functionally distinct from LMP since, from DNA sequence and RNA mapping, it should consist of only the last 258 amino acids of LMP (16).

To determine whether D1LMP is functionally distinct from LMP, we compared the activity of the two genes encoding these proteins in rodent fibroblast transformation. In the course of these studies, we also extended the previous description of LMP activity in rodent cell transformation. Further, since a significant feature of LMP in EBV-transformed lymphoblasts is its association with the vimentin cytoskeletal network (20), we examined whether LMP or D1LMP associated with the cytoskeleton in transformed fibroblasts.

MATERIALS AND METHODS

Cell culture. Rat-1 (31; obtained from R. Weinberg, Massachusetts Institute of Technology, Cambridge) or BALB/c 3T3 (obtained from the American Type Culture Collection, Rockville, Md.) cells were grown in T25 or T75 plastic flasks (Corning) in complete medium consisting of Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (KC Biologicals) and gentamicin (4 μg/ml). Subconfluent cell cultures were removed from the plastic with 0.5% trypsin-EDTA in phosphate-buffered saline and seeded at a 1:10 dilution in fresh medium.

Plasmid constructions. A fragment of the EBV genome containing the entire coding domain for the LMP message beginning 35 base pairs (bp) 5′ to the TATA box and ending 400 bp after the polyadenylation signal was cloned into pUC9 to generate pUCLM. pUCLM was cut with *Bam*HI and inserted into pHSI (19) to place the LMP gene under the control of the human metallothionein promoter. This construct was then inserted into the *Eco*RI site of the eucaryotic cell expression vector pSV2gpt (26, 29) to create pSV2gptLMP (formerly pSV2gptMTLM in reference 30)

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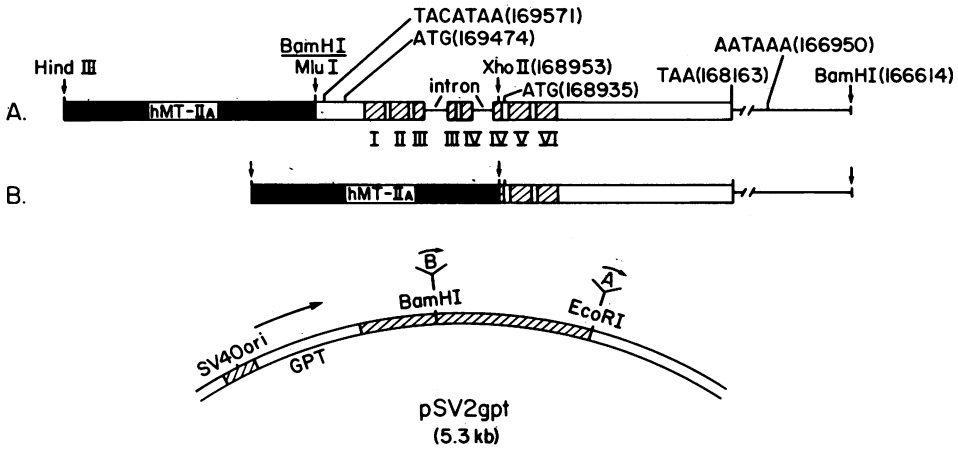

FIG. 1. Diagram of the LMP (pSV2gptLMP) and D1LMP (pSV2gptD1LMP) recombinant expression vectors. The human metallothionein promoter (filled box) was placed 35 bp 5′ to the TATA box of the LMP gene (A). The hatched boxes indicate the six putative transmembrane domains. LMP introns in the DNA which encodes the third and fourth transmembrane domains are indicated with a thin bar. The numbers in parentheses are from the published EBV DNA sequence (1). pSV2gptLMP was constructed by inserting fragment A into pSV2gpt (26, 30). To construct pSV2gptD1LMP, DNA which encodes the LMP amino terminus and the first four transmembrane domains was excised with *Xho*II. The residual LMP gene was then placed 3′ to the human metallothionein promoter to create fragment B. Fragment B was inserted into the *Bam*HI site of pSV2gpt. The transcriptional orientation of LMP and D1LMP is the same as the *gpt* gene. The hatched bars in the pSV2gpt vector indicate simian virus 40 sequence.

(Fig. 1). A 2.0-kilobase *Xho*II-*Bam*HI fragment of pUCLM (the *Xho*II site is 18 bp upstream from the ATG for the amino-terminally deleted LMP which is expressed late in EBV replication) was ligated to the metallothionein promoter and then inserted into the *Bam*HI site of pSV2gpt, using a similar cloning strategy, to create pSV2gptD1LMP (Fig. 1).

DNA transfection. Subconfluent cultures in T25 flasks (Corning), seeded 1 day previously, were transfected with 20 μg of plasmid DNA without carrier (14). In some experiments, 20 μg of pSV2gptD1LMP and 5 μg of pSV2neo (26) were used. One day later, cells were transferred into a T75 flask. After another day, mycophenolic acid (25 μg/ml), supplemented with hypoxanthine (10 μg/ml) and xanthine (150 μg/ml), or G418 (500 μg/ml) was added. At 2 to 3 weeks later, resistant foci were marked, photographed, and picked with the aid of a glass cloning cylinder (Bellco).

Soft agar cloning. Samples of 5×10^4 to 20×10^4 cells were seeded in 5 ml of medium containing 0.3% low-melting-temperature agarose (Sea-Kem, FMC Corp.), over 5 ml of medium containing 0.6% agarose, in a 60-mm petri dish. Cultures were fed with 2 drops of complete medium once a week and scored weekly.

Immunoblot analysis. Cultures were chilled on ice and scraped into 4°C PBS containing 5 mM iodoacetamide. After centrifugation, the cells were suspended in sample loading buffer (0.2 M Tris [pH 7.0], 2% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, 10% glycerol, 0.02 mM bromophenol blue) and boiled, and 3.0×10^5 cells were loaded onto a lane of a 7.5% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, the proteins were electrotransferred onto nitrocellulose paper (5). The blot was incubated sequentially at 37°C with 2.5% milk in phosphate-buffered saline (PBS), with S12 monoclonal antibody against LMP (22), with rabbit anti-mouse antibody (Cappel Laboratories), and with ^{125}I-protein A (Amersham Corp.).

Immunofluorescence staining. Cells were seeded on eight-well slides (Titer-Tek, Miles Corp.). The next day, the adhered monolayers were rinsed with PBS, immediately fixed in cold absolute methanol for 6 min, rinsed in PBS, and incubated at 37°C sequentially with 10% newborn bovine serum in PBS, with S12 monoclonal antibody, with biotinylated goat anti-mouse antibody (Bethesda Research Laboratories), and with fluorescein isothiocyanate (FITC)-streptavidin (Bethesda Research Laboratories). Cells were visualized and photographed with a Zeiss Axioskop equipped with epifluorescence with FITC and Texas Red filter sets.

For double immunofluorescence, methanol-fixed cells were stained with S12 monoclonal antibody, followed by FITC-goat F(ab′)$_2$ anti-mouse immunoglobulin G (heavy and light chain specific; Jackson Immunoresearch), and with rabbit anti-vimentin antibody (kindly provided by R. Hynes) followed by Texas Red-labeled goat anti-rabbit immunoglobulin G (heavy and light chain specific; Jackson Immunoresearch).

Detergent extraction. Cells from the confluent T25 flasks were scraped and washed in cold PBS. They were then extracted three times with PBS containing 0.5% Triton X-100 as described previously (20). The LMP and D1LMP in the extracted and insoluble fractions were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting.

RESULTS

To compare the rodent cell-transforming activity of the late virus replication cycle (amino-terminally deleted) form of LMP (D1LMP) with the transforming activity of LMP, the DNA segments encoding the D1LMP or LMP were recombined with the human metallothionein promoter and inserted into pSV2gpt (26). The *Xho*II site used to obtain the deleted DNA fragment is 18 bp 5′ to the presumed D1LMP translational initiation codon (Fig. 1). The recombinant expression vectors containing the complete or deleted LMP genes under control of the human metallothionein promoter were pSV2gptLMP and pSV2gptD1LMP, respectively.

Rat-1 cell transformation. The plasmids, pSV2gptLMP or pSV2gptD1LMP, constructed to express LMP or D1LMP, respectively, were transfected into Rat-1 cells (14). After 2

weeks in selective medium, several dozen foci were evident in each plate. In the previous series of experiments evaluating LMP transforming activity in Rat-1 cells, individual transfected cell foci were cloned and expanded into cell lines which were then assayed for contact inhibition and anchorage dependence (30). While it was apparent from these studies that LMP was a prerequisite for loss of contact inhibition and anchorage dependence, these latter phenotypic changes could have also required secondary changes in the cells which could have taken place during sequential passage in culture after transfection. So as to increase knowledge of the immediacy and directness of the LMP effect in rodent fibroblasts, in the first series of experiments the cells from each transfected flask were pooled (instead of cloning, expansion and testing of individual clones) and counted and either were plated in new flasks, to directly observe cell morphology and contact inhibition, or were seeded in soft agar to directly observe anchorage dependence. At the same passage, part of each culture was also seeded onto a plate from which average LMP expression was determined by Western blot. LMP expression in the pooled pSV2gptLMP transfectants was relatively low, while D1LMP expression in the pooled pSV2gptD1LMP transfectants was quite high (Fig. 2, middle panel; compare Rat1-LMP and Rat1-D1LMP lanes). LMP and D1LMP were the same size in Rat-1 cells as in IB4 cells, a latently infected lymphoblast line which expresses only LMP, or in B95-8 cells, a partially permissive lymphoblast line which expresses both LMP and D1LMP. With regard to cell morphology and contact inhibition, flasks of confluent pSV2gptLMP-transfected cells contained heaped-up foci of long, refractile, and narrow cells which were surrounded by a dense monolayer of swirled, narrow, somewhat less refractile cells. The appearance of these monolayers was indistinguishable from that of recently derived Rat-1 LMP clones (Rat1-LMP1 or -2 clones, Fig. 2A). In contrast, cells transfected with pSV2gptD1LMP were more square and grew as even, light monolayers, indistinguishable from cells transfected with pSV2gpt (Rat1-V, Fig. 2A). Only the pSV2gptLMP transfectants showed anchorage-independent growth in soft agar (Fig. 2C, Rat1-LMP versus Rat1-V or Rat1-D1LMP). A substantial fraction of the pSV2gptLMP-transfected cells consistently grew into macroscopically evident clumps of more than 200 cells by 2 weeks. In contrast, cells transfected with vector or pSV2gptD1LMP did not grow in soft agar beyond occasional microscopically visible clumps of between 8 and 64 cells. The soft agar cloning efficiency for the pooled pSV2gpt1LMP plasmid-containing cells, as assayed by macroscopically visible colony growth at 2 weeks, was about 2% versus 0% of visible colonies out of 10^4 pSV2gpt- or pSV2gptD1LMP-transfected cells seeded per plate (Table 1). Thus, LMP acutely transforms Rat-1 cells as assayed by loss of contact inhibition and anchorage-independent growth in soft agar, while D1LMP has no such effects.

The inefficient (approximately 2%) soft agar cloning of the pooled pSV2gptLMP-transfected Rat-1 cells could be due to the relatively low average LMP expression level if anchorage independence in these cells is a quantitative function of LMP expression (Fig. 2B, Rat1-LMP versus IB4). All cells might be expressing LMP in relatively low amounts and grow in soft agar with low efficiency, or there might be large differences among cells in LMP expression which could correlate with transformation-associated phenotypic changes. Alternatively, if there were an inverse correlation between LMP expression level and transformation, the failure of D1LMP to transform Rat-1 cells could be due to high-level expression. To evaluate these possibilities, Rat-1 cells were transfected as before and individual mycophenolic acid-resistant foci were marked, photographed, and cloned. Four heaped-up foci and four flat foci were picked, expanded, and assayed for LMP expression (Fig. 2B, Rat1-LMP1 or Rat1-LMP2 versus Rat1-LMP5 or Rat1-LMP6) for contact inhibition (Fig. 2A), or for anchorage dependence (Fig. 2C). Although mycophenolic acid-resistant foci from cells transfected in parallel with pSV2gpt vector or with pSV2gptD1LMP did not heap up, an equal number of these foci were assayed in parallel. The expression level of individual pSV2gptLMP-transfected clones varied dramatically. Clones derived from PSV2gptLMP-transfected, heaped-up foci expressed large amounts of LMP, continued to heap up in monolayer culture, and grew in soft agar (Fig. 2A, Rat1-LMP1 or -2). Clones derived from flat foci showed little or no LMP expression and behaved like normal Rat-1 cells (Fig. 2A, Rat1-LMP5 or -6). High-level LMP-expressing clones (Rat1-LMP1 or -2) grew efficiently in the soft agar (35 to 40% cloning efficiency; Table 1). pSV2gptD1LMP- or control vector-transfected foci uniformly continued to behave like normal Rat-1 cells, despite the expression of D1LMP in most of the pSV2gptD1LMP-transfected clones being similar to that of the high-level LMP expressers (Fig. 2 and Table 1). Thus, although high-level LMP expression results in Rat-1 cell transformation, high-level D1LMP expression does not.

BALB/c cell transformation. To investigate whether the transforming effects of LMP and lack of effects of D1LMP were restricted to Rat-1 cells, a similar series of studies were done in BALB/c 3T3, a continuous mouse fibroblast cell line. Low-passage BALB/c 3T3 cells were transfected with pSV2gptLMP, with pSV2gptD1LMP, or with pSV2gpt. Drug-resistant cells from each flask were pooled, counted, and replated to observe cell morphology, contact inhibition, anchorage dependence, and overall level of LMP or D1LMP expression (Fig. 3). The mycophenolic acid-resistant BALB/c 3T3 cells after pSV2gptLMP transfection were longer and thinner than pSV2gptD1LMP- or pSV2gpt-transfected cells. pSV2gptD1LMP- or pSV2gpt-transfected cells grew to a sparse monolayer with each cell separated from its neighbors. In contrast, pSV2gptLMP-transfected cells grew into a tight monolayer with cells growing across each other. Heaped-up cell clumps did not occur among transfected, resistant BALB/c 3T3 cell foci or after cloning and passage as was observed with pSV2gptLMP-transfected Rat-1 cells. However, newly transfected, first-passage, pooled, pSV2gpt LMP-transfected Rat-1 cells. However, newly transfected, first-passage, pooled, pSV2gptLMP-trasfected BALB/c 3T3 cells cloned with at least as high efficiency in soft agar as did pSV2gptLMP-transfected Rat-1 cells. By 2 weeks in soft agar, 2 to 3% of the cells had grown to macroscopically visible colonies (versus 2% of the Rat-1 transfectants; Table 1). In contrast, pSV2gptD1LMP- or pSV2gpt-transfected and mycophenolic acid-resistant cells did not grow into colonies larger than 16 cells, and no colonies were macroscopically visible at 2 weeks. On the average, pSV2gptLMP-transfected BALB/c 3T3 cells expressed slightly more LMP than did Rat-1 cells. pSV2gptD1LMP-transfected clones expressed about as much D1LMP as was previously observed with transfection of Rat-1 cells (Fig. 3).

In another set of pSV2gptLMP transfections into BALB/c 3T3 cells, 12 random, mycophenolic acid-resistant foci were cloned and expanded into cell lines. After passage in tissue culture for 3 months, they were checked for LMP expression. The clones could be generally categorized into those

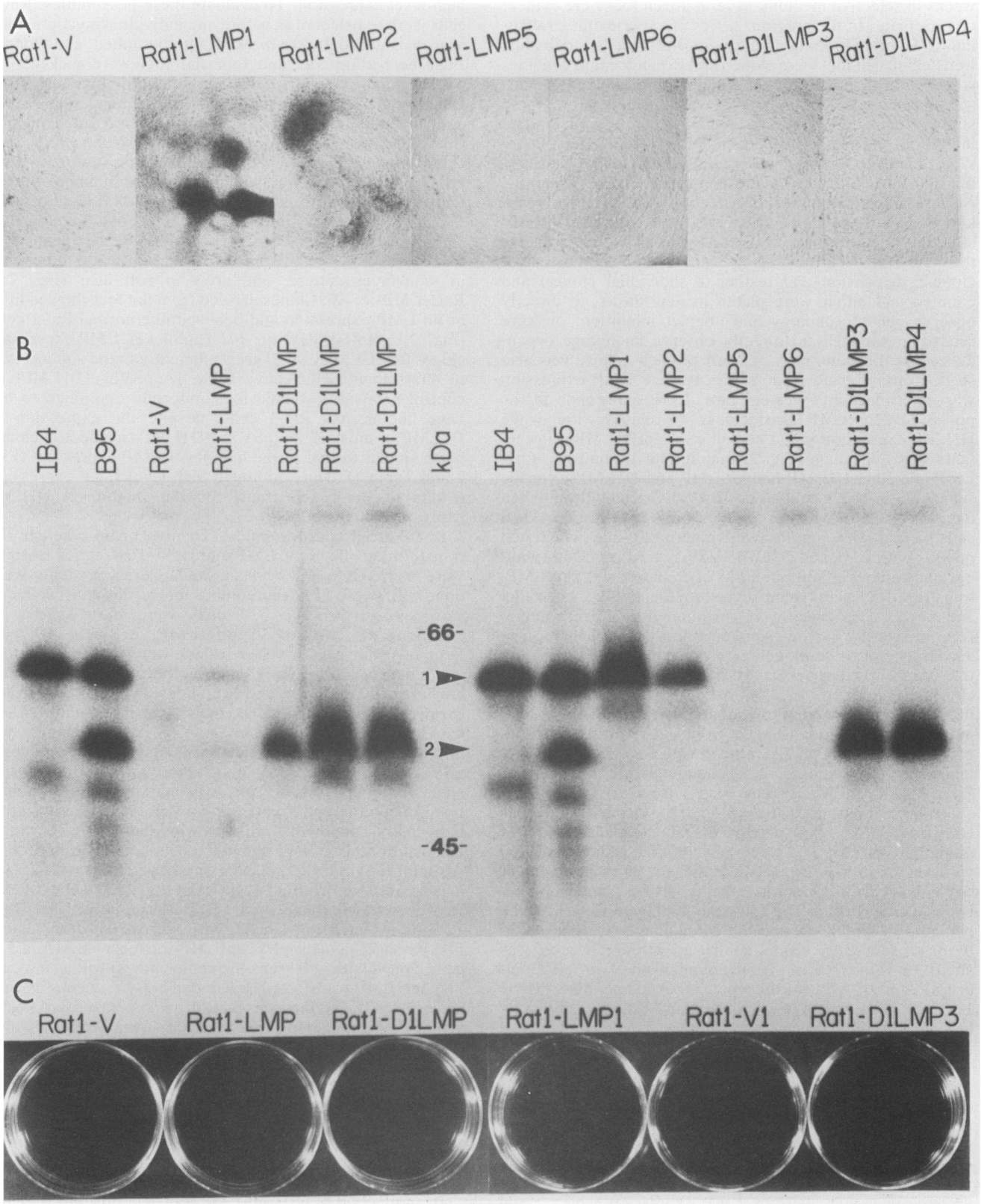

FIG. 2. Transforming effects of LMP or D1LMP on Rat-1 cells. Rat-1 cells were transfected with pSV2gptLMP or with pSV2gptD1LMP. Transfected cells were selected on the basis of their growth in mycophenolic acid and were then assayed for LMP (Rat1-LMP) or D1LMP (Rat1-D1LMP) expression, cell morphology, contact inhibition, or anchorage-independent growth in soft agar. Individual independent clones of transfected cells (indicated by a numeric suffix, e.g., Rat1-LMP1) were similarly assayed. Dust spots on the Rat-1 D1LMP, Rat1-V, or Rat1-D1LMP3 soft agar plates should not be mistaken for anchorage-independent colonies, which did not occur. IB4 is a latently infected, growth-transformed human lymphoblastoid cell line in which LMP is expressed. B95-8 (B95) is a partially productively infected marmoset cell line which expresses LMP and D1LMP. LMP is susceptible to endogenous proteases. The major LMP fragments are, however, different in size from D1LMP. kDa, Molecular size in kilodaltons. 1, LMP; 2, D1LMP.

TABLE 1. Soft agar cloning efficiency of transfected cells

Cell line	Cloning efficiency (%)[a]
Uncloned	
Rat1-V	0
Rat1-LMP	2
Rat1-D1LMP	0
Cloned	
Rat1-LMP1	35
Rat1-LMP2	40
Rat1-LMP5	0
Rat1-LMP6	0
Rat1-D1LMP3	0
Rat1-D1LMP4	0

[a] The percentage of cells growing to a colony larger than 1 mm (>200 cells) was scored two weeks after seeding in 0.3% agarose.

expressing little or none, moderate, or high levels of LMP (Fig. 4). The clones tended to remain stable in their characteristic LMP expression. One clone from each group was seeded into soft agar. Three weeks later, there were significant differences in the size of the colonies; the size of foci

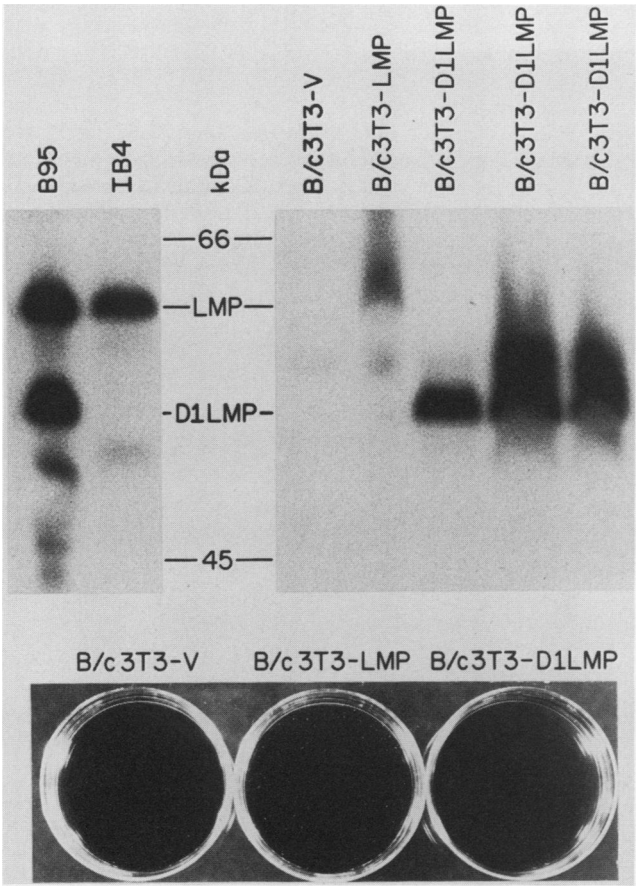

FIG. 3. Transformation effects of LMP or D1LMP on BALB/c 3T3 cells. BALB/c 3T3 cells were transfected with pSV2gpt (B/c3T3-V), pSV2gptLMP (B/c3T3-LMP), or pSV2gptD1LMP (B/c3T3-D1LMP). Resistant foci were pooled and assayed for LMP or D1LMP expression or for growth in soft agar. No macroscopically visible colonies are evident in the BALB/c 3T3-V or BALB/c 3T3 D1LMP soft agar plates. kDa, Kilodaltons.

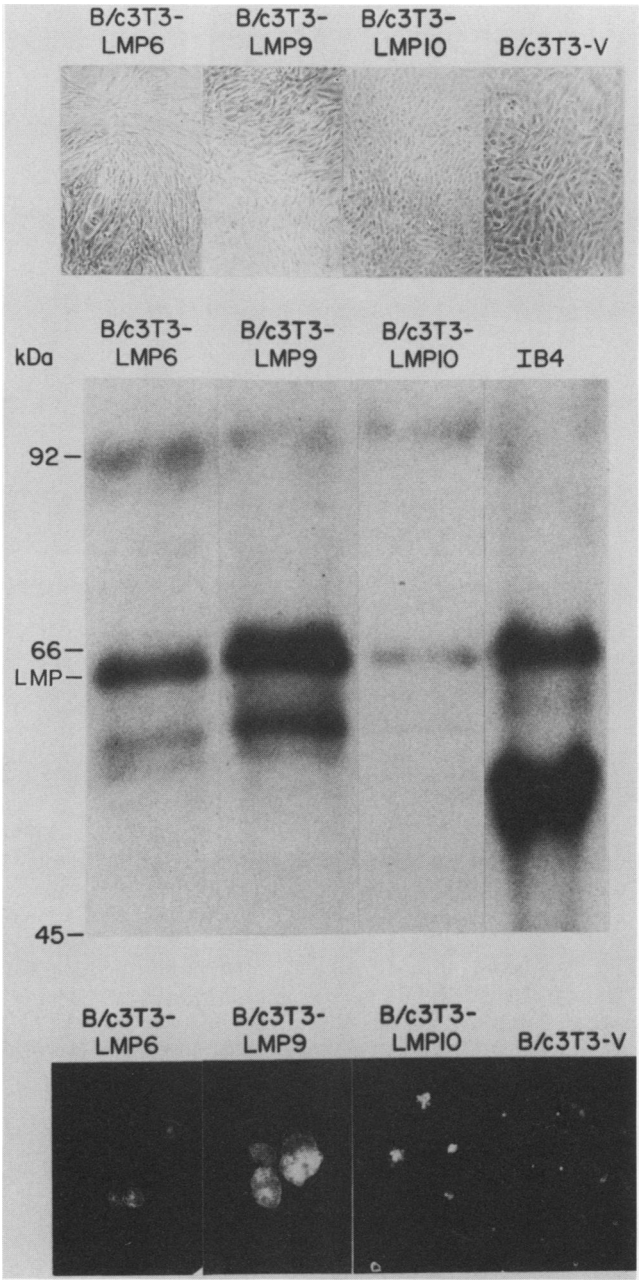

FIG. 4. Transformation of BALB/c 3T3 cells correlates with LMP expression. Clones of BALB/c 3T3 cells transfected with pSV2gptLMP which express high (B/c3T3-LMP9), middle (B/c3T3-LMP6), or low (B/c3T3-LMP10) levels of LMP are shown to exemplify characteristic changes in cell morphology, contact inhibition, and growth in soft agar (2 weeks after seeding). Colonies in soft agar plates are shown at the bottom (magnification, ×28, using a Zeiss tissue culture phase microscope). kDa, Kilodaltons.

correlated with the level of LMP expression (Fig. 4). The clones expressing moderate or high LMP levels also exhibited altered cell morphology when grown on plastic plates (BALB/c 3T3-LMP6 or -LMP9, respectively; Fig. 4). These cells appeared longer, more spindle shaped, and more refractile at the edges than low-level LMP-expressing cells, D1LMP-expressing cells, or vector-transfected control cells. There were no clear differences between the moderate- and high-level-expressing clones in their growth on plastic

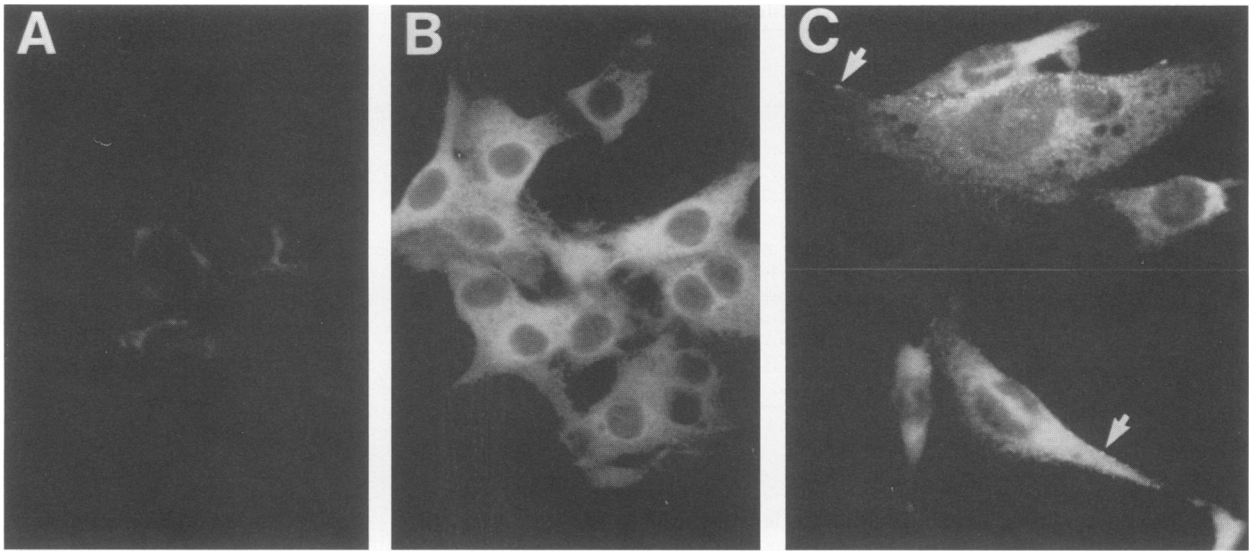

FIG. 5. Immunofluorescent staining of LMP- or D1LMP-expressing BALB/c 3T3 cells. BALB/c 3T3 cells transfected with pSV2gpt (A), pSV2gptD1LMP (B), or pSV2gptLMP (C) were stained with monoclonal antibody S12 (22), biotinylated goat anti-mouse immunoglobulin G, and FITC-streptavidin and photographed with a Zeiss fluorescent photomicroscope under epifluorescence (×1,000). Arrows indicate LMP-specific punctate membrane staining.

plates, even though there were consistent differences in LMP expression. The low-LMP-expressing cell lines (for example, BALB/c 3T3-LMP10; Fig. 4) were square shaped and grew less densely than the moderate- and high-LMP-expressing clones but could be readily distinguished from the vector-transfected control cells (BALB/c 3T3-V; Fig. 4), which were consistently squarish and evenly distributed. Thus, in BALB/c cells, there is a direct relationship between LMP expression level and transformed cell phenotype.

Localization of LMP and D1LMP in fibroblasts. Since D1LMP lacked the transforming activity of LMP, the comparative intracellular distribution of LMP and D1LMP might be relevant to the mechanisms of LMP-induced transformation. With anti-LMP monoclonal antibody S12 in indirect fluorescent microscopy, LMP-expressing Rat-1 or BALB/c 3T3 cells differed in staining patterns from D1LMP-expressing cells (30; Fig. 5). As was previously noted for LMP-expressing Rat-1 cells (30), LMP localized to the plasma membrane with specific punctate distribution and to the cytoplasm with perinuclear accentuation. D1LMP was pancytoplasmic in distribution with no evidence for plasma membrane association.

Cytoskeletal association of LMP and D1LMP in fibroblasts. LMP is associated with the vimentin cytoskeletal network in EBV-transformed lymphocytes (20). To determine whether LMP or D1LMP associates with the cytoskeleton in fibroblasts, Rat-1 cells expressing LMP or D1LMP were extracted with PBS containing 0.5% nonionic detergent (Triton X-100). The amount of LMP and D1LMP in the detergent-soluble and insoluble cell fractions was determined by immunoblotting.

Most of the LMP in transformed Rat-1 cells was extractable with buffer consisting of isotonic salt and nonionic detergent. Approximately 20% of the LMP was not extracted and remained persistently associated with the cell residue despite repeated extractions (Fig. 6). Thus LMP associates with the cytoskeleton in transformed Rat-1 fibroblasts as it does in EBV-transformed lymphocytes. In contrast, D1LMP was completely extracted from D1LMP-expressing Rat-1 cells and was not detectable in the detergent-insoluble fraction (Fig. 6). Thus, D1LMP is not tightly associated with the cytoskeleton.

To determine whether LMP associates with vimentin in Rat-1 cells, clones of LMP-expressing Rat-1 cells were stained by double immunofluorescence for LMP and vimentin. Due to the high-level expression and nearly pancytoplasmic distribution of vimentin in these cells, it was often difficult to assess whether LMP and vimentin colocalized. However, isolated vimentin fibers could occasionally be distinguished which stained for both LMP and vimentin (Fig.

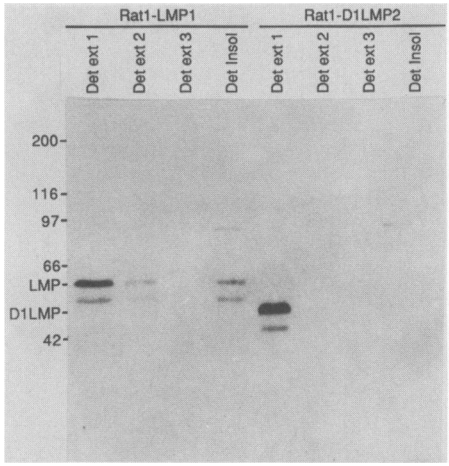

FIG. 6. Differential cytoskeletal association of LMP or D1LMP in Rat-1 cells. Rat-1 cells expressing LMP (Rat1-LMP1) or D1LMP (Rat1-D1LMP2) were extracted three times with 0.5% Triton X-100 under physiological salt conditions (PBS–0.5% Triton X-100). The LMP or D1LMP in the detergent-soluble and detergent-insoluble fractions was analyzed by Western immunoblotting. Lanes: Det ext 1, 2, and 3, first, second, and third detergent extractions, respectively; Det Insol, detergent-insoluble cytoskeletal residue. LMP and D1LMP are indicated at the left.

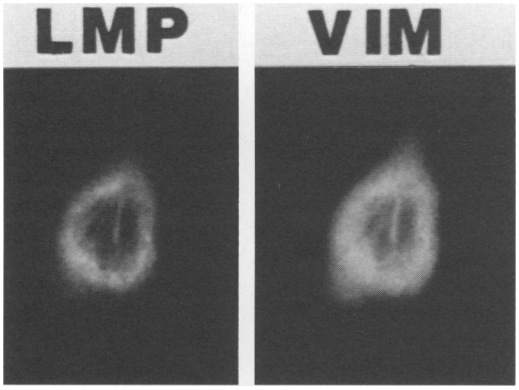

FIG. 7. LMP and vimentin colocalization in Rat-1 cells. The Rat-1 cell clone Rat1-LMP2 was stained by double immunofluorescence as described in Materials and Methods. Fluorescein anti-LMP (LMP) and Texas Red anti-vimentin (VIM) staining is shown in the same cell. Note the double staining of filaments over the nucleus.

7, over the nucleus). Usually, LMP was in discrete dots along vimentin filaments at the cell periphery. (These could not be captured in photographs.) D1LMP staining never colocalized to vimentin filaments, even though the vimentin filaments were stained in D1LMP-expressing cells (data not shown). These data, along with the detergent extraction data, demonstrate that LMP is associated with the vimentin cytoskeletal network in transformed fibroblasts.

Effects of LMP and D1LMP coexpression. Even though LMP and D1LMP are made during different stages of EBV infection in lymphocytes and D1LMP was inactive in transforming rodent cells, D1LMP has the last two transmembranes and the entire carboxy-terminal acidic cytoplasmic LMP domains, either of which could be important interactive sites between LMP and cell macromolecules. D1LMP could therefore inhibit or augment LMP effects in cell transformation. We therefore examined the possible interaction between LMP and D1LMP in the Rat-1 system. A low-passage LMP-transformed Rat-1 cell clone (Rat1-LMP2) was cotransfected with the pSV2gptD1LMP plasmid and pSV2neo at a ratio of 4:1, and G418-resistant cells were selected. We expected that we would get both D1LMP-positive and D1LMP-negative, G418-resistant, LMP-expressing Rat-1 cell foci, providing us with an ideal internal control. We marked, photographed, and cloned the resultant foci, which differed in the extent to which they heaped up in culture. Eleven foci were expanded into lines. These were then counted and seeded in soft agar and checked for LMP and D1LMP protein expression. Positive and negative D1LMP-expressing clones were observed, but there was also variation in LMP expression with some LMP-negative clones (Fig. 8). In agreement with the previous results, LMP expression correlated with anchorage independence (Fig. 8 and Table 2). D1LMP expression did not correlate with this effect. Interestingly, the simultaneous expression of D1LMP did not change the phenotype of LMP-positive Rat-1 cells. For example, clone pairs 8 and 9 or 11 and 12 expressed similar levels of LMP, with the second member of each pair also expressing D1LMP. In both instances, the LMP-plus-D1LMP-expressing clone was indistinguishable from the LMP-expressing clone in loss of contact inhibition and in anchorage independence. The finding that D1LMP did not inhibit LMP transformation could be due to the insignificance of the D1LMP domains to the interaction of LMP with the cell, to inappropriate D1LMP intracellular distribution, or to the putative D1LMP cellular interaction being with a nonlimiting cellular factor.

DISCUSSION

In the experiments described here, the amino-terminally deleted D1LMP, expressed late in the EBV replication cycle, consistently lacked rodent cell-transforming activity. Thus, the rodent cell-transforming activity of full-length LMP correlates with its expression in latent, growth-transforming EBV infection, and the lack of rodent cell-transforming activity of D1LMP correlates with its expression late in EBV replication. These data are consistent with the working hypothesis that rodent cell transformation is reflective of the role of LMP in EBV-induced human lymphocyte growth transformation.

Based on mRNA mapping (16) and nucleotide sequence (1, 12), D1LMP translation is predicted to begin at a methionine near the end of the fourth of the six LMP transmembrane domains. This is consistent with the relative size of LMP and D1LMP in denaturing polyacrylamide gels. D1LMP would therefore lack the hydrophilic LMP amino terminus (4 arginines, 2 glutaminic acids, and 1 aspartic acid of 20 residues) and the first four transmembrane domains, including the first reverse turn which is exposed on the outer cell surface, but would share with LMP two other transmembrane domains and a 210-amino-acid, hydrophilic, acidic (20% aspartic or glutamic acid), cytoplasmic carboxy-terminal domain (12, 15, 20, 21). Although the last two transmembrane domains common to LMP and D1LMP are probably adequate to assure membrane association, the multiple membrane-spanning domains of LMP could be necessary for LMP activity if it is a channel protein as are several other proteins having multiple membrane-spanning domains, such as the sodium channel or glucose transporter proteins (25, 27).

A potentially important correlate of the different activity of LMP and D1LMP in rodent cell transformation and in EBV-induced lymphocyte transformation is the association of LMP with the rodent fibroblast or human lymphocyte cytoskeleton. In particular, in EBV-infected human lymphocytes, EBV associates with vimentin intermediate filaments (20). In latently EBV-infected, growth-transformed lymphocytes, vimentin is drawn into LMP-associated patches at the cell periphery. A small fraction of the LMP expressed in rodent fibroblasts is also at the cell periphery and is in small patches (Fig. 5; 30). These multiple small LMP aggregates in Rat-1 or BALB/c 3T3 fibroblasts are also associated with vimentin, although the large amount of vimentin intermediate filaments in those rodent fibroblasts, most of which do not associate with LMP, results in an excessive background which prevents us from photographically documenting the association except in those instances where isolated vimentin filaments with bound LMP could be located. The correlation between the cytoskeletal association and transformation associated with LMP and the lack of both activities in D1LMP is consistent with a model in which (vimentin) cytoskeletal association is integral to LMP transforming activity both in lymphocytes and in fibroblasts. The simplest explanation for the difference between LMP and D1LMP in cytoskeletal association and transforming activity (and for the failure of D1LMP to inhibit LMP transforming activity) is that the LMP amino-terminal cytoplasmic domain, missing from D1LMP, mediates cytoskeletal association and directly plays a role in transformation. More complex hypotheses could include roles for the amino terminus or first four

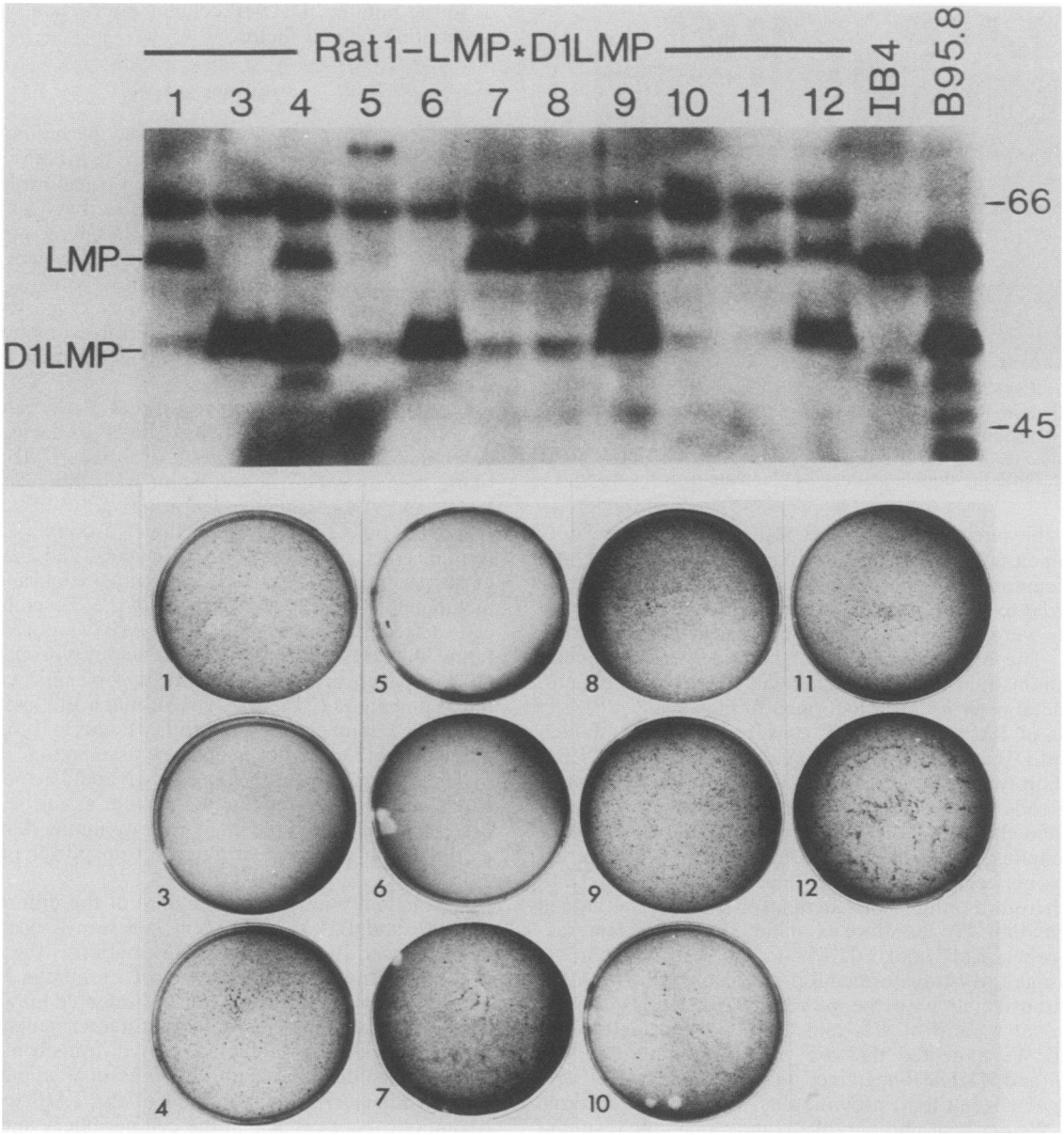

FIG. 8. Effects of LMP and D1LMP coexpression in Rat-1 cells. The Rat1-LMP2 clone was cotransfected with pSV2neo and pSV2gptD1LMP. Eleven G-418-resistant foci were marked, photographed (plates 1 through 3), and cloned. Each clone was assayed for LMP or D1LMP expression and for growth at 1 week in 0.3% agarose.

transmembrane domains in LMP posttranslational modification (2, 20) or intracellular compartmentalization (which could, in turn, affect cytoskeletal association), and directly or indirectly affect cell transformation. In lymphocytes, LMP is processed before becoming associated with the cytoskeleton (20). Complex interactions might be anticipated, on the basis of recent experiments with $pp60^{src}$ and *erbB* mutations, in that mutations which affect membrane localization did not fully block affects on cell proliferation or transformation (3, 7–10, 13, 18). Importantly, the new data on LMP activity and D1LMP inactivity in rodent fibroblast transformation not only are consistent with the working hypothesis that LMP effects in the rodent fibroblast transformation system are relevant to EBV-induced human lymphocyte growth transformation, but they also indicate that the LMP rodent fibroblast transformation model is susceptible to molecular genetic and molecular biologic analysis.

These experiments significantly extend previous observations regarding EBV LMP activity in rodent fibroblast transformation (31). We have for the first time demonstrated that LMP expression in BALB/c 3T3 (or in Rat-1) cells acutely and efficiently causes altered cell shape, increased refractility, loss of contact inhibition, and anchorage independence. Thus, the effects of LMP on rodent fibroblast transformation do not require prolonged cell passage for their manifestation (6). Further, a quantitative relationship has been demonstrated between extent of LMP expression and BALB/c cell transformation, as has been previously demonstrated with $pp60^{src}$ (17) and polyomavirus middle T antigen (28).

LMP induction of BALB/c 3T3 cell anchorage indepen-

TABLE 2. Interaction between LMP and D1LMP in Rat-1 cells

Clone no.	Morphology[a]	LMP expression[b]	D1LMP expression[c]	Soft agar cloning efficiency (%)[d]
1	+	++	−	27
3	−	−	++++	2
4	++	++	++++	27
5	−	−	−	1
6	−	−	++++	4
7	++	+++	−	86
8	++	+++	−	90
9	+	+++	++++	28
10	−	+	−	27
11	+	++	−	47
12	++	++	++++	27

[a] −, Large, square shaped cells growing in a monolayer; ++, long spindly, refractile cells growing densely and heaping up; +, phenotype intermediate between − and ++.
[b] Relative level or absent (−) LMP expression.
[c] D1LMP expression relative to LMP expression or absent (−).
[d] One week after seeding, colonies with more than 200 cells were scored as positive. Percentage of cells giving rise to colonies was determined by scoring and averaging four randomly chosen fields.

dence indicates that the different anchorage-dependent phenotypes previously observed in NIH 3T3 and Rat-1 cells are likely due to the specific interactions of LMP with each of these immortalized cells, perhaps in complementing the specific establishment function of each cell type, and are not due to an inherent inability of LMP to induce anchorage-independent growth in murine cells. In support of this hypothesis, that each immortalized cell genotype idiosyncratically complements LMP, are the observations that LMP also induces different levels of release from contact inhibition in NIH 3T3, BALB/c 3T3, or Rat-1 cells. Thus, LMP-expressing BALB/c 3T3 or Rat-1 cells frequently grew across each other, while LMP-expressing NIH 3T3 cells did not, even when LMP was expressed in NIH 3T3 cells at high levels under the control of the Moloney murine leukemia virus promoter (31). Furthermore, LMP-expressing BALB/c 3T3 cells rarely, if ever, heaped up when grown on plastic surfaces as did LMP-expressing Rat-1 cells, despite overall higher levels of LMP expression in some of the BALB/c cells and a slightly higher cloning efficiency of the LMP-expressing BALB/c cells in soft agar. The different LMP interactions with each of these immortalized cell lines are potentially useful in analyzing cellular components of the transformation phenotypes. For example, a molecular genetic approach of transferring BALB/c cell and LMP DNA to a cell line such as NIH 3T3, which has low background soft agar cloning efficiency, might lead to identification of a BALB/c 3T3 gene which complements LMP in anchorage independence, if the LMP-complementing BALB/c 3T3 gene were to be phenotypically dominant in NIH 3T3 cells.

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