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Myristylation of Rous Sarcoma Virus Gag Protein Does Not Prevent Replication in Avian Cells

CHRISTINE R. ERDIE AND JOHN W. WILLS*

Department of Biochemistry and Molecular Biology, 1501 Kings Highway, P.O. Box 33932, Louisiana State University Medical Center—Shreveport, Shreveport, Louisiana 71130

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Rous sarcoma virus is an example of a replication-competent retrovirus whose Gag protein is not modified with myristic acid. The purpose of the experiments described in this report was to determine whether the addition of this 14-carbon fatty acid would interfere with the replication of Rous sarcoma virus. We found that myristylated derivatives of the Rous sarcoma virus Gag protein are fully functional for particle formation in avian cells and that the addition of myristic acid has very little effect on infectivity.

The production of retroviral particles by budding from the plasma membrane of the host cell requires only one viral gene product, the Gag protein (4, 5, 19, 29). The Gag proteins of most retroviruses have a 14-carbon fatty acid, myristate, attached to their amino termini (16, 18, 24). Well-characterized examples of these include the Gag proteins of murine leukemia virus (14), Mason-Pfizer monkey virus (15), and human immunodeficiency virus (1). With no known exceptions, myristic acid is attached cotranslationally (11, 28) through an amide bond to a glycine residue following removal of the initiator methionine (8). Alteration of the myristic acid addition site by site-specific mutagenesis abolishes particle formation (1, 14, 15), demonstrating the importance of this fatty acid for Gag function among these retroviruses. The actual role of myristate in Gag function is not understood, though it has generally been thought to be involved in plasma membrane interactions at the site of assembly.

Those few retroviruses whose Gag proteins function without the need for myristate are quite diverse and include the avian sarcoma-leukosis viruses (17), equine infectious anemia virus, and visna virus of sheep (16, 24). The range of host species represented by these retroviruses overlaps that of the myristylated viruses, arguing that modification is not an absolute requirement for replication in any particular type of cell. This idea is further supported by the existence of myristylated retroviruses, such as reticuloendotheliosis virus (17, 25) and the xenotropic and amphotropic strains of murine leukemia virus (23), which are capable of replicating in a very broad range of host species.

We have been investigating the Gag protein of Rous sarcoma virus (RSV; 22), $Pr76^{gag}$, which is among those that are not myristylated (17). Although this Gag protein drives particle formation with high efficiency in avian cells, it had long been thought not to do so in mammalian cells (26, 27). However, recently we have shown that $Pr76^{gag}$ actually can function in mammalian (CV-1) cells when expressed at a level similar to that obtained in an authentic RSV infection in avian cells (29). This observation further indicates that the presence or absence of myristate is not correlated with Gag function in particular host species. Nevertheless, our studies also demonstrated that the RSV Gag protein acquires a greatly enhanced ability to form particles in mammalian cells when myristate is added to its amino terminus (29). Sites for addition have been created in two ways. In $Pr76^{myr1}$, the first 10 amino acids of the Gag protein are replaced with those of $pp60^{src}$, another myristylated protein which is targeted to the plasma membrane (2, 9, 12). In $Pr76^{myr2}$, the glutamic acid residue following the initiator methionine is changed to glycine by means of a single nucleotide change. It is not known why myristylation enhances the ability to form particles in simian cells, but the effect is dramatic.

Having found that myristate does not inhibit, but enhances, RSV Gag function in mammalian cells, we were curious to see how $Pr76^{myr1}$ and $Pr76^{myr2}$ would behave in avian cells. In particular, we wanted to determine whether the addition of myristic acid would diminish the infectivity of RSV. Such an effect might be expected, since RSV and its relatives in the avian sarcoma-leukosis virus family have evolved without the need for myristate, even though a single nucleotide change is sufficient to create a site for addition on $Pr76^{gag}$ (29).

Expression of $Pr76^{myr1}$ in avian cells. The most direct way to test the effects of a *gag* mutation on virus replication is to exchange it for the wild-type sequence in the RSV genome and to monitor the infectivity of the recombinant upon transfection into avian cells. Although this approach was suitable for analyzing the *myr2* allele (see below), it is nonsensical for *myr1* because that mutation destroyed the splice donor site, located in the first 10 codons of *gag*, which is required for the synthesis of *env* mRNA and thus infectivity (20). Nevertheless, we wanted to determine whether $Pr76^{myr1}$ is capable of producing particles in avian cells and therefore decided to express it in the absence of Pol and Env proteins. Because our previously described simian virus 40-based vectors do not express in avian cells (data not shown), we placed the *myr1* allele under the control of the murine leukemia virus long terminal repeat promoter, which does. The vector chosen for these experiments was pDOL.ATG− (kindly provided by Constance Cepko, Harvard University), a derivative of pDOL− (10, 13) in which the initiation codon of the residual murine leukemia virus *gag* sequence has been destroyed.

The *myr1* allele was excised from pSV.Myr1 (29) by using *Sac*I and *Bss*HII, purified by agarose gel electrophoresis, and ligated into pDOL.ATG− at its unique *Sal*I site after the DNA ends were made blunt by using the Klenow fragment of DNA polymerase I. The plasmids were propagated in *Escherichia coli* DH-1 and selected on Luria-Bertoni agar plates

* Corresponding author.

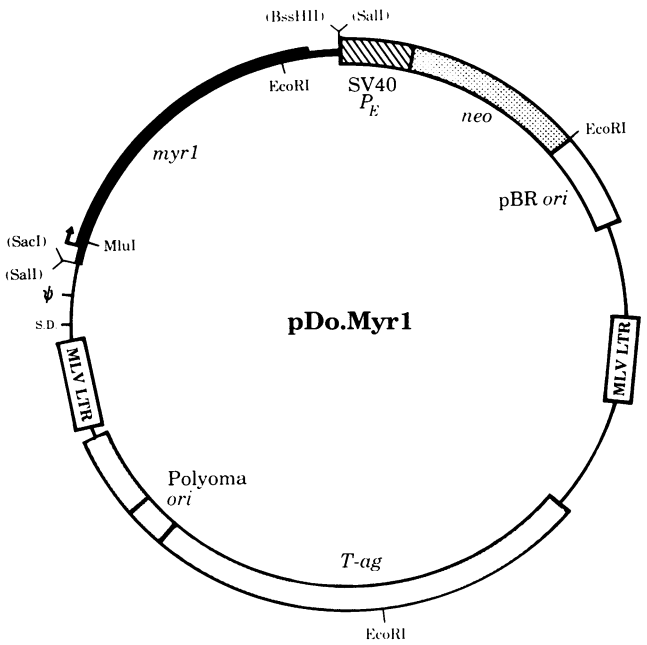

FIG. 1. Vector for expression of Pr76^{myr1} in murine and avian cells. The *myr1* allele is represented by the thick black line in which the start of the coding sequence is marked with an arrow. The unique *Mlu*I site was created while altering *gag* to encode the first 10 amino acids of p60$^{v\text{-}src}$. The *Sac*I-*Bss*HII fragment was cloned from pSV.Myr1 into the murine leukemia virus vector at the *Sal*I site, thereby destroying the sites indicated in parentheses. The leftmost long terminal repeat (LTR) serves as the promoter for transcription of *myr1*. The simian virus 40 virus early promoter (P_E) drives transcription of the neomycin resistance gene which is selected in *E. coli* by using kanamycin. The polyomavirus origin of replication (*ori*) and gene for T antigen (*T-ag*) allow amplification in murine cells. pDo.Gag is identical to this plasmid except that it contains the wild-type RSV *gag* gene.

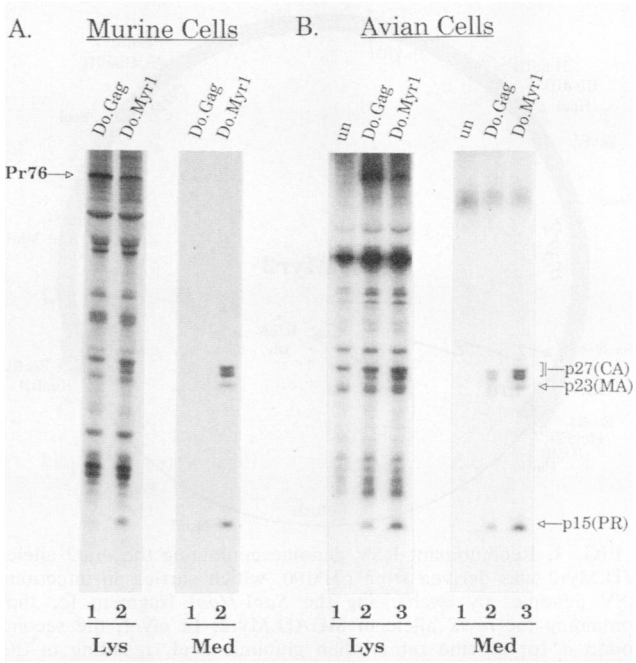

FIG. 2. Expression of Myr1 proteins in murine and avian cells. (A) Murine (3T3) cells were transfected with pDo.Gag (lanes 1) or pDo.Myr1 (lanes 2) and labeled 48 h later with [^{35}S]methionine for 2 h. The Gag proteins were immunoprecipitated from the cell lysates (Lys) and culture media (Med) with anti-RSV antibodies, electrophoresed in an SDS–12% polyacrylamide gel, and detected by fluorography. Positions for Pr76^{gag}, p27(CA), p23(MA), and p15(PR) are indicated. (B) Turkey embryo fibroblasts were transfected with no DNA (un; lanes 1), pDo.Gag (lanes 2), or pDo.Myr1 (lanes 3). The cells were subsequently labeled, and RSV Gag proteins were immunoprecipitated and analyzed in the same manner as that used for transfected murine cells.

containing kanamycin (25 μg/ml). A recombinant bearing the *myr1* allele in the proper orientation relative to the long terminal repeat promoter was obtained and named pDo.Myr1 (Fig. 1). A control plasmid in which the wild-type *gag* gene has been inserted into the vector at the identical position is named pDo.Gag (also provided by the laboratory of Constance Cepko).

Our initial characterizations of pDo.Gag and pDo.Myr1 were carried out by transient expression assays in murine (NIH 3T3) cells. Dishes (35 mm) of 3T3 cells were transfected with 5 μg of DNA, using the DEAE-dextran method followed by a chloroquine treatment, as described previously (29). Two days after transfection, the cultures were labeled with [^{35}S]methionine for 2.5 h and partitioned into medium and cell lysate fractions. The labeled RSV Gag proteins were immunoprecipitated from the detergent-treated samples using rabbit anti-RSV antibodies, separated by electrophoresis in sodium dodecyl sulfate (SDS)–12% polyacrylamide gels, and detected by fluorography, as described before (29).

On the basis of our previous experiments using simian cells, we expected that the unmyristylated product of pDo.Gag would produce particles at a very low level and that the myristylated product of pDo.Myr1 would have enhanced activity; this prediction was found to be accurate (Fig. 2A). For both constructs, a Gag precursor of about 76 kilodaltons was detected in the cell lysates, and that produced by pDo.Gag seemed to accumulate to a greater extent than that of pDo.Myr1 (lysate lanes 1 and 2, respectively). This accumulation presumably is due to the reduced ability of the pDo.Gag product to form particles relative to that of the pDo.Myr1 product (compare medium lanes 1 and 2). The Gag-related proteins present in the Myr1 particles correspond to the mature cleavage products derived from Pr76 (MA, CA, NC, and PR). The same cleavage products were observed in the medium from the cells transfected with pDo.Gag; however, these bands are barely visible on the exposure presented in Fig. 2. Untransfected cells and cells transfected either with pDOL.ATG− or with a plasmid bearing the *gag* gene in the wrong orientation revealed only background bands (data not shown).

Having shown in murine cells that functional *gag* alleles had been inserted into pDo.Myr1 and pDo.Gag, we repeated the transient expression assays using turkey embryo fibroblasts (Fig. 2B). Once again, the Gag precursor produced by pDo.Gag accumulated in the cells at a higher level than that for pDo.Myr1 (lysate lanes 2 and 3). However, relative to the murine results, the level of particle formation obtained with pDo.Gag was more like that of pDo.Myr1 (medium lanes 2 and 3). To be sure that these particles were not the result of an endogenous virus, an uninfected control which proved to be negative was included (lysate and medium lanes 1). The higher level of particle formation by the pDo.Gag product in avian cells relative to mammalian cells is not surprising,

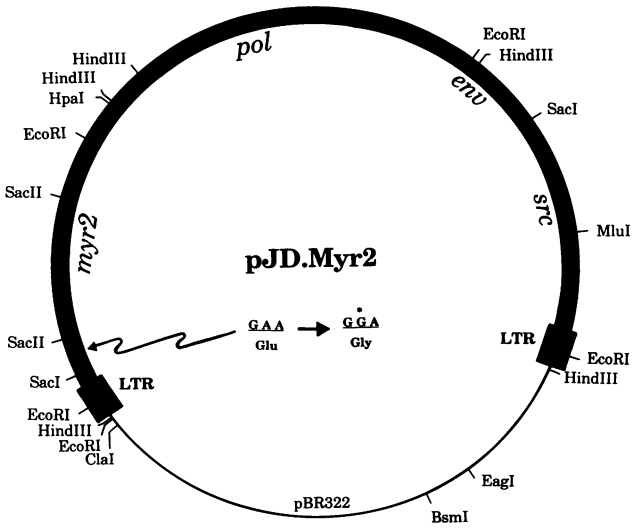

FIG. 3. Recombinant RSV genome containing the *myr2* allele. pJD.Myr2 was derived from pJD100, which carries an infectious RSV genome, by exchanging the *Sac*I-*Hpa*I fragment for that containing the *myr2* allele in MGAG.Myr2. In *myr2*, the second codon is for glycine rather than glutamic acid, resulting in the synthesis of a myristylated Gag protein. The squiggly arrow indicates the approximate position of the *myr2* mutation (*) within the RSV genome.

since this Gag protein is identical to that of wild-type RSV. What is surprising is that the Myr1 protein produces higher levels of particles than the wild-type protein in its native cell type. We do not understand why this myristylated Gag protein is such a consistently efficient producer of particles; however, this characteristic should prove useful for analysis of RSV Gag function in a variety of cell types.

Expression of Pr76^{myr2} in avian cells. As mentioned above, the *myr2* mutation does not alter the splice donor site located at the 5′ end of the *gag* coding region, so it was reasonable to use this allele to study the effect of myristylation on RSV infectivity. The source of the *myr2* sequence was the replicative form DNA of MGAG.Myr2, the M13mp19 clone that was originally created by mutagenesis. The gene was isolated after digestion with *Sac*I and *Hpa*I and inserted into the place of the wild-type sequence in pJD100, a pBR322 derivative containing an infectious copy of the RSV Prague A genome (7, 21), to create pJD.Myr2 (Fig. 3). Because the *myr2* mutation was created in the Prague C *gag* gene, we also constructed a control plasmid containing the parent allele, and this was named pJD.Myr0.

Turkey embryo fibroblasts were transfected with pJD100, pJD.Myr0, or pJD.Myr2 as described elsewhere (29). Three days later, all three cultures simultaneously showed early signs of morphological transformation which continued to develop at the same rate over the next few days until all of the cells in the monolayer were transformed (data not shown). The development and spread of transformation by RSV can occur in this manner only if the transfecting DNA is infectious (3). Thus, these results demonstrate that the *myr2* mutation is not detrimental to RSV replication.

To confirm that the Myr2 protein was myristylated in the avian cells, as it is in mammalian cells (29), monolayers infected with JD100 or one of two clones of JD.Myr2 were radiolabeled with [^{3}H]myristic acid. As a control, duplicate cultures were labeled with [^{35}S]methionine to detect unmyristylated proteins. The labeling period was limited to 10 min

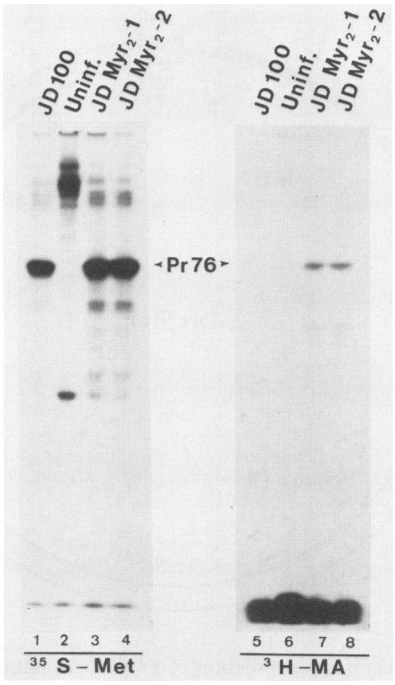

FIG. 4. Labeling of JD.Myr2-infected turkey cells with [^{3}H]-myristic acid. Uninfected cells (lanes 2 and 6) or cells infected with pJD100 (lanes 1 and 5), pJD.Myr2 clone 1 (lanes 3 and 7), or pJD.Myr2 clone 2 (lanes 4 and 8) were labeled for 10 min with [^{35}S]methionine (left panel) or [^{3}H]myristic acid (right panel). Gag proteins were immunoprecipitated from cell lysates using anti-RSV antibodies and electrophoresed in an SDS–12% polyacrylamide gel prior to detection by fluorography. Bands near the top of the gel correspond to fibronectin.

to prevent the metabolic conversion of the [^{3}H]myristic acid into tritiated amino acids (and subsequent labeling of non-myristylated proteins), a process that occurs very rapidly in RSV-transformed cells (data not shown). Gag proteins in the cell lysates were immunoprecipitated and analyzed by gel electrophoresis, as described above. That the pJD100- and pJD.Myr2-infected cells were producing Gag proteins is demonstrated by the results of the [^{35}S]methionine labeling (Fig. 4, left panel, lanes 1, 3, and 4). These Gag precursors could not have been produced by viruses endogenous to the turkey cells, because Pr76-related proteins were not detected in the uninfected control (lane 2). The results of [^{3}H]myristic acid labeling (Fig. 4, right panel) showed that the Myr2 proteins produced by both clones of JD.Myr2 were indeed myristylated in avian cells (lanes 7 and 8). This labeling could not be due to isotopic conversion, since the Gag protein of JD100 (lane 5) was not labeled. As expected, the Gag protein of JD.Myr0 was not labeled either (data not shown).

From the semiquantitative experiments described above, we could not detect any differences in the replication rates of JD100, JD.Myr0, and JD.Myr2, and we conclude that myristylation of the RSV Gag protein must not dramatically impair infectivity. To examine this more quantitatively, we compared the accumulation of reverse transcriptase activity in the medium of cells infected with the three viruses. Each type of virus was freshly prepared from fully transformed turkey cells and diluted to the same concentration on the basis of reverse transcriptase activity. Identical amounts of each virus were used to infect 60-mm plates of uninfected

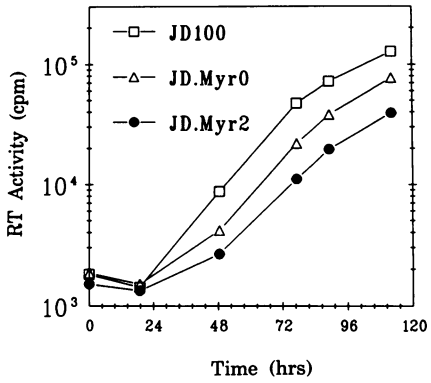

FIG. 5. Accumulation of reverse transcriptase activity in the medium of infected cells. Identical plates of turkey embryo fibroblasts were infected with identical amounts (on the basis of reverse transcriptase activities) of freshly prepared virus of the three types indicated. After the infected cells were washed thoroughly, fresh medium was added, and small samples were then removed at the indicated times postinfection for measurement of reverse transcriptase (RT) activity.

cells for 2 h in the presence of Polybrene (2 μg/ml). After infection, the plates were washed several times before the addition of 5 ml of fresh medium, from which small samples were collected at various times over the next 5 days. Reverse transcriptase assays were performed by standard methods (6) after all the samples had been collected.

As expected, the results showed that all three strains of virus were capable of rapidly producing particles (Fig. 5). However, the results also revealed that JD.Myr2 is slightly impaired in its ability to accumulate in the medium. This slight reduction has been observed consistently in repeated experiments and with both clones of JD.Myr2 (data not shown). To some extent, this minor difference appears to arise as a result of exchanging the Prague A *gag* gene with that from the Prague C genome, since we consistently observed an intermediate rate of particle accumulation with JD.Myr0 (Fig. 5). We have confirmed the results of the reverse transcriptase experiment by labeling infected cells with [^{35}S]methionine and measuring the relative levels of Gag protein released into the medium. Setting JD100 at 100%, we found that JD.Myr0 and JD.Myr2 release particles at 93 and 80% of this level, respectively (data not shown).

From the results presented in this report, we conclude that the infectivity of JD.Myr2 is basically that of wild-type RSV, despite the addition of myristic acid to the amino terminus of Pr76^{gag}. At this point, we do not know what accounts for the slight reduction in particle formation that we consistently observe. While it is quite possibly due to the presence of myristic acid, it might instead derive from an alteration at the RNA level (e.g., a slight change in the efficiency of RNA splicing) caused by the single base substitution. We have not grown JD.Myr2 through multiple rounds of replication to determine whether a selective pressure exists for loss of the myristic acid addition site, and we have not compared various strains of RSV to see to what extent the levels of particle formation vary among wild-type isolates.

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