Parvovirus Replication in Normal and Transformed Human Cells Correlates with the Nuclear Translocation of the Early Protein NS1

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The parvovirus H-1 infection of the normal human diploid fibroblast strain MRC-5 produces a cytopathic effect, but no increase in infectious virus has been observed. Previously, we reported that large amounts of empty capsids are assembled in the nucleus of H-1 infected MRC-5 cells (S. Singer and S. Rhode, *in* D. Ward and P. Tattersall, ed., *Replication of Mammalian Parvoviruses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1978). The level of viral replicative-form DNA synthesis as shown by metabolic labeling is markedly reduced in these cells. Synthesis of the early protein NS1 is normal or slightly decreased, and the usual amount of the 92,000-molecular-weight (92K) posttranslationally modified NS1 was seen. The second deficient parameter that we have observed in the abortive infection is the nuclear translocation of NS1. In contrast, the simian virus 40-transformed MRC-5 cell line MRC-5 V1 and the simian virus 40-transformed human kidney cell line NB undergo a productive infection by H-1 accompanied by more efficient translocation of NS1 to the nucleus. The results indicate that there is an association between defective translocation of the NS1 rep protein to the nucleus and defective amplification of parvovirus replicative-form DNA. The nuclear translocation of specific proteins seems to be a function that is altered by development or neoplastic transformation.

Parvoviruses have small single-stranded DNA genomes of about 5,000 nucleotides that contain two or three structural genes. Each gene produces several related gene products as a result of different mRNA splicing options (for a review, see references 3, 9, and 44). The genetic simplicity of these viruses implies a high degree of dependence on host cell functions for replication, and many examples of host cell restrictions to productive infection have been described previously (1, 2, 5, 7, 11, 12, 22, 38, 39). The first viral synthetic event is synthesis of the complementary DNA strand, and this process appears to be S-phase dependent (29, 45). This requirement can explain the dependency of the autonomously replicating parvoviruses on replicating cells for infection. The parvoviruses show a preference for fetal cells or renewal tissues *in vivo* and replicate most productively in fetal cells or transformed cells *in vitro* (9). The failure of parvoviruses to replicate in many types of normal cells *in vitro* cannot be ascribed to the virus dependency on S-phase-specific functions but must result from other restrictions for required host cell functions. We have characterized one of these abortive infections, H-1 infection of MRC-5 fibroblasts and their simian virus 40 (SV40)-transformed counterparts. We previously observed that H-1 infection of MRC-5 or WI38 human diploid fibroblasts was nonproductive for infectious virus, but a net increase in virus could be obtained from the SV40-transformed WI38 VA13 cell line (P. Paradiso and S. Rhode, unpublished results). Similar results were reported for human diploid fibroblasts WI26 (20) and for secondary cultures of diploid human lung fibroblasts and embryonic kidney cells (19). The restriction could be partially relieved by coinfection with adenovirus 12 (19). Because the MRC-5 fibroblasts replicated in culture more rapidly than WI38 or WI26 and could be infected at a high

efficiency with H-1, we selected this cell line for further study. Ultrastructural studies revealed the accumulation of large amounts of empty capsids in the nucleus of H-1-infected MRC-5 fibroblasts (38). In some cases, permanent mouse or rat cell lines can be enhanced for their susceptibility to cell killing by parvoviruses after transformation with SV40 (5), polyoma middle T gene, or the activated human Harvey-*ras*-1 oncogene (22). A more recent report describes the enhanced susceptibility of human fibroblasts to H-1 or minute virus of mice infection after transformation to permanent cell lines with ionizing radiation, chemical carcinogen, or SV40 (7). The nonstructural early protein NS1, or *rep*, of parvoviruses plays a pivotal role in regulating virus replication. NS1 *trans*-activates the promoter for capsid gene expression (17, 33, 35, 42) and is required for viral DNA replication (6, 10, 14, 21, 32, 41, 42). Recent evidence indicates that NS1 is the protein that is found covalently bound to the 5' termini of viral DNA (10, 26, 43). The other nonstructural protein of the rodent autonomous parvoviruses, NS2, has no known function. We have examined the abortive infection of MRC-5 fibroblasts and characterized the synthesis of NS1, its posttranslational phosphorylation, and its intracellular localization. The most outstanding difference between the abortive and permissive infections is the poor nuclear localization of NS1 in the abortive infection.

MATERIALS AND METHODS

Cells and virus strains. MRC-5 (human diploid fibroblasts) ATCC CCL 171, NB (SV40-transformed human newborn kidney) (36), and MRC-5 V1 and V2 (SV40-transformed MRC-5 [15]) cells were propagated in monolayer culture with Eagle minimal essential medium supplemented with glutamine and 5 to 10% fetal calf serum. The MRC-5 V1 and V2 cell lines were the kind gift of Robin Holliday. H-1 virus was propagated in NB cells, and virus purification (24), H-1 replication-form (RF) DNA preparation (30), radiolabeling of viral DNA (29), viral hemagglutination, and plaque assays

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(20) were as previously described. Parasynchronous cultures of NB cells or subconfluent MRC-5 cells were prepared with a methotrexate block as described previously (28).

Gel electrophoresis. Horizontal gel electrophoresis of DNA was carried out in 1% agarose as described previously (31). The radioactivity in DNA bands which were located by autoradiography was quantitated by excising the appropriate area of the gel, digesting the slice in 0.15 ml of 50 mM Tris hydrochloride (pH 7.8)–5 mM MgCl₂–5 µg of DNase I per ml for 16 h at 37°C, solubilizing with 0.6 ml of NCS solubilizer (Amersham), and assaying for radioactivity by liquid scintillation spectrometry. The DNase digestion facilitated extraction of ³H- or ³²P-nucleotides from the gel slice and avoided undetected adsorptive quenching.

Immunoprecipitations and protein gel analysis were done by the method of Laemmli (18), and fluorography was done by the method of Bonner and Laskey (4) as described previously (25).

Immunofluorescent staining. NS1 was visualized by indirect immunofluorescence as previously described by using the rabbit anti-NS1 antisera provided by Sue Cotmore (8, 34).

RESULTS

H-1 replication in MRC-5 cells and SV40-transformed MRC-5 fibroblasts. Infection of MRC-5 cells in asynchronous growth at a cell concentration of about $2 \times 10^4/\text{cm}^2$ or 30 to 50% confluency resulted in 95% of the cells showing bright intranuclear immunofluorescence for H-1 capsid antigens at 24 h postinfection (p.i.). MRC-5 cultures infected at a multiplicity of infection (MOI) of 10 to 20 showed a definite cytopathic effect when viewed in the inverted microscope, as characterized by an initial retraction of cell processes and rounding of cells, an increase in refraction of light at the cell margins, and finally degeneration of the cell layer and cell death (data not shown). Only one colony-forming cell (out of 10^6 cells) survived infection with wild-type H-1 at 37°C at an MOI of 10 to 20. Therefore, nearly all of the MRC-5 cells are susceptible to infection by H-1.

The synthesis of H-1 hemagglutination (HA) was not defective in MRC-5 cells, but no increase in infectious virus was observed at various times postinfection up to 72 h. A similar result was obtained with the parvovirus LuIII, which was originally isolated from a human cell line (37). In contrast to MRC-5 cells, the SV40-transformed MRC-5 cell lines MRC-5 V1 and MRC-5 V2 supported productive infections with H-1 (data not shown). These results are similar to those recently described by Cornelis et al. (7).

Synthesis of H-1 RF DNA and progeny DNA in MRC-5 cells. The synthesis of H-1 RF DNA and progeny DNA, extracted from infected cells by the method of Hirt (13), was compared in MRC-5 and NB cells. Viral DNA was labeled with ³²P_i (and [³H]thymidine in experiment 2) from 12 to 16 h p.i., and replicate cultures were processed for determination of HA synthesis and immunofluorescent staining for H-1 capsid antigens. The Hirt supernatants containing the labeled viral DNA were fractionated by agarose gel electrophoresis. The gels were dried, the regions containing viral DNA were identified by autoradiography and excised, and the ³²P and ³H were measured by liquid scintillation spectrometry. A representative autoradiogram is illustrated in Fig. 1, and the results are summarized in Table 1. When the numbers of infected cells (FA positive) are normalized, the yield of H-1 monomer RF DNA in MRC-5 cells is very low, 1 to 2% of that from NB cells. The amount of label at the position of

FIG. 1. Agarose gel electrophoresis of Hirt extracts of metabolically labeled H-1-infected MRC-5 cells and NB cells. The infections and labeling are as described in the legend to Table 1. The marker RF DNA is sucrose gradient-purified RF DNA labeled with ³²P_i from NB cells, and virion DNA was similarly labeled and extracted from purified virions.

progeny DNA relative to RF DNA is increased in MRC-5 cells compared with that in NB cells. The cause of this difference between relative yields of RF DNA and progeny DNA is unknown. Similar results were obtained when the experiment was done with nonsynchronized cells. It can be concluded from these experiments that progeny DNA synthesis is present in MRC-5 cells, but we did not detect that the progeny DNA that is synthesized becomes properly

TABLE 1. Synthesis of H-1 DNA in MRC-5 fibroblasts and NB cells^a

Expt and cell line	RF DNA (cpm)	ss-DNA (cpm)	log ₂ HA	FA ^b
1				
NB	62,260	8,420	18	69
MRC-5	960	2,060	14	37
MRC-5/NB	0.02	0.23	0.12	1.0
2				
NB	127,950	11,260	17	30
MRC-5	1,070	1,190	12	17
MRC-5/NB	0.01	0.1	0.06	1.0

^a The yields of ³²P_i-labeled RF DNA (monomer) and single-stranded viral DNA (ss-DNA) were determined for H-1-infected MRC-5 fibroblasts and NB cells in two separate experiments. Parasynchronous cultures of NB cells or MRC-5 fibroblasts were infected with H-1 at an MOI of 20 and labeled with ³²P_i (50 µCi/ml) in a low-phosphate medium at 12 to 16 h p.i. In experiment 2, the cultures were also labeled with [³H]thymidine and the results were similar (data not shown). Viral DNA was extracted by the Hirt method and fractionated in an 1% agarose gel for 560 V × h (Fig. 1). The ³²P contents of the bands of viral DNA were measured as in Materials and Methods. Twice as many MRC-5 cells as NB cells were used for labeling viral DNA. Replicate cultures were harvested for HA determination and immunofluorescent (FA) staining for H-1 capsid protein.

^b FA values are the mean numbers of positive nuclei per microscopic field at 400 × (10 fields counted) at 16 h p.i. Thus, FA values represent a relative concentration of infected cells per unit area at the time the viral DNA was extracted.

FIG. 2. (A) Gel electrophoresis of purified empty capsids of wild-type H-1 isolated from infected NB cells or infected MRC-5 fibroblasts. The infected cells and virus were collected at 72 hours p.i. Conditions of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis were as previously described (24). (B) Gel electrophoresis of $[^{35}\text{S}]$ methionine-labeled and immunoprecipitated viral proteins from MRC-5 cells infected with H-1. MRC-5 cells or NB cells were infected with H-1 at an MOI of 100 and metabolically labeled for 2 h preceding sample collection as previously (25). Shown are H-1 infected NB cells at 15 h p.i. (lane 1) and H-1 infected MRC-5 cells at 12 (lanes 2 and 6), 18 (lanes 3 and 7), 24 (lanes 4 and 8), and 32 (lanes 5 and 9) h p.i. Lanes 1 through 5 were immunoprecipitated with convalescent hamster anti-H-1 antiserum, and lanes 6 through 9 were immunoprecipitated with guinea pig anti-capsid antiserum. — and *—, NS1 84K and 92K bands, respectively.

encapsidated into virions (data not shown). More importantly, the amplification of RF DNA is markedly deficient in MRC-5 cells.

Synthesis of H-1 proteins in MRC-5 cells. The increase in HA titer in MRC-5 cells infected with H-1 demonstrates the synthesis of capsid protein during infection. Previous electron microscopic analysis of H-1-infected MRC-5 cells showed that capsids were formed although they appeared to aggregate (as compared with infected NB cells) (38). Analysis of purified empty capsid protein from MRC-5 infected cells (Fig. 2A) showed the expected viral capsid proteins VP1 and VP2' at the same size and in the same proportion as seen for the capsids produced in NB cells.

The noncapsid virus protein NS1 was also studied by analyzing H-1-infected cell lysates as done previously for H-1-infected NB cells (25). As seen in Fig. 2B, the two forms of NS1, 84,000-molecular weight (84K) and 92K, were synthesized during the infection of MRC-5 cells. The NS1 proteins are identified by their reactivity with sera from neonatally infected hamsters and not with anti-capsid antiserum (compare Fig. 2B, lanes 1 and 6). Also of interest in Fig. 2B is the appearance of high-molecular-weight forms of the capsid proteins late in the infection (lanes 5 and 9). These findings correlate with the electron microscopy data showing aggregate formation in infected cells. Labeling of NS1 with $^{32}\text{P}_i$ was also found to occur in H-1-infected MRC-5 cells as in NB cell infections (data not shown).

Cellular localization of NS1. We recently observed as an incidental finding that the cellular localization of NS1 was

abnormal in HeLa cells. A majority of the cells did not translocate NS1 to the nuclei. Therefore, we examined the H-1-infected MRC-5 fibroblasts for the cellular localization of NS1 by indirect immunofluorescence. The results are illustrated in Fig. 3A and 3B. In a majority of the infected cells (70 to 80%), the cytoplasm is brightly fluorescent and NS1 appears to be excluded from the nucleus. In some cells, nuclei are clearly positive, but the concentration gradient of signal between the nucleus and the cytoplasm is not as marked as in permissive cells such as NB (compare Fig. 3B to D). H-1-infected HeLa cells show several different types of cellular localization of NS1 (Fig. 3C). There is a predominance of cells with a diffuse cytoplasmic localization, some cells with a discrete focal deposition of NS1 in the perinuclear area, and some cells with normal nuclear localization. This correlates with the finding that H-1 replicates poorly in HeLa cells (S. Rhode, unpublished observation). In contrast, the infected MRC-5 V1 cells show a much more efficient translocation of NS1 to the nucleus (Fig. 4). We scored 83% of the infected MRC-5 V1 cells positive for nuclear fluorescence at 16 h p.i., and the remainder had predominantly cytoplasmic fluorescence.

DISCUSSION

The autonomously replicating parvoviruses replicate without a helper virus, but they are very dependent on host cell functions for many steps in their replication. Some of these steps can be revealed by analysis of the many types of

FIG. 3. Micrographs of indirect immunofluorescent staining for NS1 by using the monospecific anti-NS1 rabbit antisera. Cultures were fixed for staining at 24 h p.i. (A) MRC-5 fibroblasts with an infected cell with cytoplasmic fluorescence and an uninfected cell (arrow). (B) MRC-5 fibroblasts showing both cells with cytoplasmic and cells with nuclear and cytoplasmic localization of NS1. (C) H-1-infected HeLa cells with three types of NS1 localization as discussed in the text. Panel (D) H-1-infected NB cells (permissive) with a high degree of localization of NS1 to the nucleus. Magnification, $\times 870$.

restricted infections where complete virus replication does not occur (1, 2, 5, 7, 11, 12, 22, 38, 39). In this study, we have characterized the abortive infection of MRC-5 fibroblasts by the parvovirus H-1. H-1 efficiently infected replicating MRC-5 cells, but production of infectious virus was not detectable. Capsid proteins were synthesized at normal levels, and they assembled into empty virions that appeared to be the same as those seen in productive infections (38). Capsid protein synthesis by translation *in vitro* of H-1 mRNA extracted from MRC-5 cells was similar to that of NB cell viral mRNA (data not shown). The aggregation of

capsids in MRC-5 cells *in vivo* may correlate to the apparent aggregation of viral proteins from late in infection that was seen during gel electrophoresis (38). This aggregation may be due to an abundance of capsid protein synthesis in the absence of progeny DNA encapsidation. This type of protein aggregation is seen on occasion late in productive infections of NB cells with H-1 (P. Paradiso, unpublished results).

The most outstanding defect in the abortive infection of MRC-5 fibroblasts is a very deficient amplification of viral RF DNA, which is 1 to 2% of that seen in a productive infection. Transformation of these cells to a permanent cell

FIG. 4. Micrograph of indirect immunofluorescent staining for NS1 in H-1-infected MRC-5 V1 cells. Cultures were infected at an MOI of 20 and fixed for staining at 20 h p.i. Magnification, $\times 500$.

line by SV40 virus resulted in cells that can be productively infected as previously described (7). We were not able to supply a helper function for productive infection of MRC-5 cells with H-1 with adenovirus 12, as previously described for other normal human cells (19, 20; S. Rhode and N. Ledinko, unpublished data). We can infer from the abundant synthesis of capsid protein that the parental RF DNA is synthesized in the normal fashion, and this has been directly confirmed (7). Because NS1 is known to be required for viral RF DNA amplification (rep function) (6, 10, 14, 21, 32, 41, 42), we suspected that NS1 was involved in the defective RF DNA replication seen in normal human cells. However, we found that NS1 was produced in these unsynchronized MRC-5 cells simultaneously with the capsid protein in both the 84K and the highly phosphorylated 92K forms and in normal-appearing amounts at 12 h p.i. (relative to capsid proteins). At later times, the relative level of NS1 synthesis may have been decreased. Immunofluorescent localization of NS1 revealed that the nuclear translocation of NS1 was very definitely abnormal in H-1-infected MRC-5 cells. The majority of the cells showed cytoplasmic fluorescence with no immunofluorescence in the nuclei, and some cells had some weak staining of the nuclei along with cytoplasmic staining. It is possible that cytoplasmic NS1 has a more rapid turnover, so that defective nuclear translocation may result in reduced total amounts of NS1. The other nonstructural

protein of H-1, NS2, has not been assigned a function, and it was not observed in this study. Most antisera derived from infected neonatal hamsters, such as that which we used in this study, do not immunoprecipitate NS2. It is possible that NS2 synthesis or function is also defective in MRC-5 cells.

We hypothesize that efficient replication of viral RF DNA may require large amounts of NS1 in the nucleus and that inefficient nuclear translocation of NS1 is responsible for the abortive infection of normal human diploid fibroblasts. Possibly, large amounts of nuclear NS1 are necessary because there are other sites that compete for NS1 binding and competitively inhibit its participation in viral DNA replication. In a productive infection, only a small proportion of the total NS1 is associated with viral DNA (10). In support of this hypothesis, transformation of these fibroblasts to a permanent cell line (MRC-5 V1) converts the cells to a phenotype that is permissive for productive infection and is associated with an enhancement of nuclear translocation of NS1. The efficiency of nuclear translocation of NS1 in the MRC-5 V1 cells appears to be intermediate to that of MRC-5 cells and NB cells. The amounts of infectious virus produced by MRC-5 V1 cells and HeLa cells are also reduced compared with that produced by NB cells. Cornelis et al. (7) made a detailed study of viral DNA replication and infectious virus production in a number of normal and transformed human cells. Our results are in agreement with

theirs, and we suggest that there is a strong correlation between the effectiveness of the nuclear translocation of NS1 and the level of amplification of viral DNA. Amplification of viral RF DNA may be a necessary, but not a sufficient, condition for the production of high titers of virus by the infected cell. Evidence has been reported that NS1 is the protein that is covalently bound to the 5' ends of viral RF DNA (10). This interaction suggests that NS1 may be the site-specific nuclease hypothesized to be required for the hairpin transfer process that is known to occur on viral RF DNA (9). These interactions would also imply that there is a specific binding site for NS1 on viral DNA in or adjacent to the inverted terminal repeats.

Nuclear translocation of large proteins is known to be a highly specific function in eucaryotic cells. The small nuclear location peptide on the SV40 large T antigen can be inactivated by a single amino acid change (16). MRC-5 cells are productively infected by herpes simplex viruses and are known to import many viral proteins to the nucleus, such as parvovirus capsid proteins (38) and SV40 large T antigen (Rhode, unpublished data). In this study, we report evidence that the nuclear translocation of viral proteins may not only be specific for each viral protein (parvovirus NS1 versus capsid), but it can also be under developmental regulation by the cell. There is a correlation between transformation of the normal human fibroblast to a permanent cell line and the nuclear translocation of H-1 NS1. It is interesting that this is not the first report of developmental regulation of nuclear translocation of a parvovirus protein. Tattersall (40) has previously reported that the nuclear translocation of minute virus of mice capsid protein is deficient in embryonal carcinoma cells and that this restriction is relieved in at least some of the differentiated derivatives of these cells. It is possible that MRC-5 cells are deficient in a posttranslational modification of NS1 that is required for its importation to the nucleus. Since both the 84K and 92K forms of NS1 were synthesized, we have no evidence of this. Our immunofluorescent localization of NS1 did not show an accumulation at the nuclear membrane, so we assume that the binding of NS1 to a receptor at the nuclear pore, rather than the translocation step, is probably defective (23, 27). Clearly, the nuclear translocation of NS1 by the cell uses a function borrowed by the virus which is normally used to transport unidentified cellular proteins. It is potentially important that this transport mechanism shows alterations in association with cell transformation.

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