Susceptibility of Human Cells to Killing by the Parvoviruses H-1 and Minute Virus of Mice Correlates with Viral Transcription

J. J. CORNELIS,^{1*} Y. Q. CHEN,² N. SPRUYT,¹ N. DUPONCHEL,¹ S. F. COTMORE,³ P. TATTERSALL,^{3,4}
AND J. ROMMELAERE^{1,2}

Unité d'Oncologie Moléculaire, Institut Pasteur de Lille, Institut National de la Santé et de la Recherche Médicale U186 and Centre National de la Recherche Scientifique 1160, 59019 Lille Cédex, France¹; Laboratoire de Biophysique et Radiobiologie, Département de Biologie Moléculaire, Université Libre de Bruxelles, B1640 Rhode St. Genèse, Brussels, Belgium²; and Departments of Laboratory Medicine³ and Human Genetics,⁴ Yale University School of Medicine, New Haven, Connecticut 06510

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Human fibroblasts and epithelial cells differing in their susceptibility to killing by the autonomous parvoviruses H-1 and minute virus of mice were compared for their capacity to express viral mRNAs and proteins. The transition from a parvovirus-resistant to a parvovirus-sensitive phenotype correlated with a proportional increase in the production of the three major viral transcripts and of structural and nonstructural proteins. In contrast, cell sensitization to parvovirus could not be correlated with detectable changes in virus uptake, intracellular localization of gene products, stability of viral mRNAs, or phosphorylation of viral nonstructural polypeptides. Moreover, the H-1 virus-sensitive keratinocyte line studied did not sustain a greater level of viral DNA amplification than its resistant derivative. Therefore, the differential susceptibility of the human cells tested to parvovirus infection appears to be mainly controlled at the level of transcription of the viral genome. Parvoviral gene expression could not be elevated by increasing the input multiplicity of infection in either of the cell systems analyzed. Together, these data suggest that a cellular factor(s) regulating parvoviral transcription may be modulated by oncogenic transformation or by differentiation, as both features have been shown to affect cell susceptibility to parvoviruses.

Viruses containing linear single-stranded DNA genomes approximately 5,000 nucleotides in length flanked by short terminal hairpin structures belong to the *Parvoviridae* (46). The cell types that are able to sustain the replication of parvoviruses are limited by the high requirements of these viruses for exogenous helper functions. This restriction is most obvious for the subgroup of adeno-associated viruses, which only replicate either in the presence of helper viruses or in cells exposed to peculiar synchronization or stress treatments (56). On the other hand, the replication of so-called autonomous parvoviruses can take place under less-stringent conditions, although it still remains S-phase dependent and requires appropriate differentiation states (30, 47, 48).

The genomes of the autonomous rodent parvoviruses minute virus of mice (MVM) and H-1 are organized in a very similar way (13). Two overlapping transcription units produce three major cytoplasmic mRNA species (24, 39). The transcripts R1 and R2 are both initiated from the left-handed promoter (P4) at map unit 4 and translated into the nonstructural proteins NS-1 and NS-2, respectively (13, 24, 39). The P38 promoter (at map unit 38) programs R3 transcripts of approximately similar size that are generated by differential splicing and encode the capsid proteins VP-1 and VP-2 (21, 22, 32). NS-1 is a multifunctional nuclear phosphoprotein involved in viral DNA synthesis (11, 13, 29, 51). The NS-1 polypeptide also regulates positively (14) and negatively (our unpublished results) its own promoter and exerts both positive and negative effects on the transcriptional activity of the P38 promoter (15, 41, 44). Furthermore, NS-1 seems to suppress the activity of a series of heterologous promoters (44). The latter feature and the failure to establish cell lines

that constitutively express high levels of the NS-1 protein led to the suggestion that this polypeptide may be cytotoxic and contribute to the lytic effect of parvoviruses (23, 35, 42). No known functions have as yet been ascribed to the smaller NS-2 polypeptide, although evidence suggests that NS-2 may act synergistically with NS-1 in cytotoxicity (6).

In most systems studied so far, sensitization of host cells to parvovirus-induced killing proved to correlate with an increase in their capacity for parvovirus replication. Thus, oncogenic transformation of a series of human and murine cells was accompanied, on the one hand, by an enhancement of their sensitivity to the cytotoxic action of H-1 or MVM parvoviruses and, on the other hand, by a stimulation of intracellular steps of the parvoviral life cycle (9, 10, 16, 17, 52). Similarly, allotropic variants of MVM have been isolated and could be distinguished by their ability to both replicate and cause cytopathic effect in cells belonging to distinct differentiation lineages (49). Moreover, resting cells both resist parvoviruses and undergo a cryptic infection (37). Together, these data suggest that a major determinant of the sensitivity of cells to parvoviral attack is their ability to produce viral cytotoxic factors, although their intrinsic responsiveness to such factors may also be modulated (45).

The physiological state of host cells can affect various steps of the parvoviral life cycle, including uptake (27, 47), DNA replication (3, 7-9, 55), and expression (3, 10). The present study was undertaken in this framework as an attempt at defining more precisely which step(s) of parvovirus replication controls the susceptibility of human fibroblasts and keratinocytes to H-1 virus and MVMp (prototype strain of MVM). Both viruses are able to replicate in a variety of in vitro-transformed or tumor-derived human cells while infection of corresponding normal cells is abortive (7-9, 16, 17, 43, 50). In this work, parvovirus replication was

* Corresponding author.

monitored in related human cell lines consisting of a series of normal and transformed fibroblasts that are resistant and sensitive, respectively, to H-1 and MVMp viruses (9) and the H-1 virus-sensitive established keratinocyte line HaCaT10 (7) and its virus-resistant derivative HaCaT10R2. In both systems, cell susceptibility to virus-induced killing proved to correlate positively with steady-state levels of parvoviral transcripts and ensuing viral proteins. The accumulation of viral mRNAs appears to be controlled, at least in part, at the transcriptional level.

MATERIALS AND METHODS

Cells and viruses. Nonestablished lines of normal human fibroblasts (VH10, VH25, and KMS-6), immortalized preneoplastic lines of in vitro-transformed human fibroblasts (VH10SV, KMST-6, SUSM-1, WI38CT-1, and MRC5V1), and the fibrosarcoma-derived cell line HT1080 have been described previously (7, 9). VH25 and KMS-6 cells displayed the same susceptibility to MVMp and were used interchangeably. Human fibroblasts were grown in Eagle minimal essential medium supplemented with nonessential amino acids and 10% fetal calf serum. The cell line HaCaT10 is a subclone of the established human keratinocyte cell line HaCaT (5). The clone HaCaT10R2 was derived from HaCaT10 after two successive infections with H-1 virus at 10 PFU/cell, as described previously (33). Human keratinocytes were grown in Joklik modified low-calcium minimal essential medium supplemented with nonessential amino acids and 10% fetal calf serum.

Cells were synchronized by the double thymidine blockage method (1). Briefly, keratinocytes were incubated for 24 h in minimal essential medium containing 7.5 mM thymidine, washed thoroughly to get rid of the excess thymidine, and cultured for 14 h in normal medium. Cells were incubated again for 24 h in medium containing thymidine, washed as above, and infected with H-1 virus.

H-1 virus and MVMp were propagated in and counted by plaque assays on NB-E and A9 cells, respectively. Micrococcal nuclease-treated and CsCl gradient-purified full particles of both viruses were used for all infections.

RNA extraction, transfer, and hybridization. Cytoplasmic and nuclear fractions were obtained after lysis of the cells in 0.1 M Tris–0.15 M NaCl (pH 8.0) containing 1% Nonidet P-40, and RNA from each fraction was purified as described by Maniatis et al. (28). Total cellular RNA was obtained as described previously (10). In some experiments, polyadenylated RNA was selected by oligo(dT)–cellulose chromatography. For Northern (RNA) blotting, RNA samples were denatured at 95°C in the presence of formamide, matched for their rRNA contents, electrophoresed in 1.2% denaturing agarose gels, transferred to Hybond-N (Amersham Corp., Amersham, England) nitrocellulose filters, and hybridized to ³²P-labeled probe DNA as suggested by the manufacturer. The plasmid pMM984, which contains the entire MVMp genome in pBR322 (29), and plasmid pULB3514, which consists of the 2,845-base-pair (bp) *Bgl*II fragment of H-1 virus DNA integrated in the *Bam*HI site of plasmid pUC12, were used as probes for the respective parvoviruses. The blots were autoradiographed with Kodak XOMAT-S films at –70°C.

Viral RNA stability was studied by adding 8 µg of dactinomycin (Boehringer, Mannheim, Federal Republic of Germany) per ml of medium at 18 h postinfection (p.i.). Infected cultures were incubated for up to 5 h in the presence of the drug. This dose of dactinomycin was found to inhibit overall

de novo RNA synthesis more than 95% as determined by incorporation of radiolabeled uridine into cellular RNA.

RNase protection assays were performed by a modification of the method described by Zinn et al. (57). The *Hae*III-*Xho*I fragment from pMM984 was inserted between the *Sal*I and *Sma*I sites of the vector pGEM-3 (Promega Biotec, Madison, Wis.). Probe RNA was synthesized from the SP6 promoter in the presence of [α-³²P]UTP by standard methods; 5 µg of cytoplasmic RNA extracted from virus- or mock-infected cells was mixed with 2 × 10⁶ cpm of probe RNA, denatured at 85°C for 10 min, and hybridized at 45°C overnight. RNA was precipitated in ethanol, redissolved in 80% formamide–40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.4]–400 mM NaCl–1 mM EDTA and digested with both RNase A (40 µg/ml) and RNase T₁ (2,000 U/ml) in 350 µl of 10 mM Tris hydrochloride–5 mM EDTA–300 mM NaCl, pH 7.5, at 0°C for 30 min. The mixture was first treated with proteinase K (140 µg/ml) and 1% sodium dodecyl sulfate (SDS) at 37°C for 10 min, followed by phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) extraction and ethanol precipitation. Samples were redissolved in denaturing conditions, electrophoresed on 8% polyacrylamide gels containing 8.3 M urea, and autoradiographed.

Measurement of viral DNA amplification by hybridization. Total viral DNA amplification was determined by dispersed-cell assays (9). In short, 5 × 10⁵ H-1 virus-infected cells were trapped on nitrocellulose filters, denatured, neutralized, and hybridized against ³²P-labeled, nick-translated probe DNA of plasmid pULB3514. After hybridization, the filters were washed, dried, and counted. Viral DNA amplification gives the ratio of intracellular H-1 virus DNA contents at 30 h versus 2 h p.i., for a multiplicity of infection of 0.1 PFU/cell.

Protein immunoprecipitation and analysis by polyacrylamide gel electrophoresis. Cells (10⁶) were metabolically labeled for 1 h in methionine-free medium supplemented with 0.2 mCi of [³⁵S]methionine (800 Ci/mmol; Amersham Corp., Amersham, England). Cells were lysed in RIPA buffer (10 mM Tris hydrochloride [pH 7.4], 1 mM disodium EDTA, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1% aprotinin) and count-matched samples (4 × 10⁶ cpm of acid-precipitable material) were treated with appropriate antisera. The immunoprecipitates were rinsed and processed for polyacrylamide-SDS gel electrophoresis as described previously (10). The viral capsid proteins VP1 and VP2 were recognized with rabbit antisera raised against empty MVMp or H-1 capsids. The NS-1 and NS-2 nonstructural proteins were precipitated with rabbit sera directed against bacterial fusion proteins containing NS-1- or NS-2-specific amino acid sequences (12). The immunoprecipitated proteins were separated on 10% (NS-1, VP1/VP2) or 15% (NS-2) polyacrylamide-SDS gels and analyzed by fluorography. Relative amounts of proteins were quantified by densitometry (CDS-200-Beckman). Acid phosphatase assays on immunoprecipitated proteins were performed as described by Pognonec et al. (40).

For the metabolic labeling with ³²P_i, cultures were preincubated for 15 h in phosphate-free minimal essential medium, followed by a 2-h incubation in the same medium supplemented with 2 mCi of ³²P_i (10 mCi/ml; Amersham Corp., Amersham, England). The cells were processed for immunoprecipitation (10) in the presence of 50 mM NaF.

Indirect immunofluorescence. H-1 virus (5 PFU/cell) or mock-infected cells were fixed at 18 h p.i. with paraformaldehyde and processed as described previously (52). Cells were first treated with specific antiserum at a dilution of

TABLE 1. Growth rate and H-1 virus susceptibility of human keratinocyte clonal derivatives

Cell clone	Population doubling time (h)	Virus adsorption ^a (%)	Viral DNA amplification ^b	Cell survival ^c (%)
HaCaT10	29	20	1.1 ± 0.3	2 ± 5
HaCaT10R2	29	21	1.0 ± 0.6	90 ± 5

^a Determined as described by Chen et al. (7).^b Determined by dispersed-cell assays as described in Materials and Methods (average values ± standard deviations from three experiments).^c Residual clonogenicity on plastic of virus-infected (10 PFU/cell) versus mock-treated cells (average values ± standard deviations from three experiments).

1:100 for 30 min, rinsed with phosphate-buffered saline, further incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G, and examined by fluorescence microscopy.

RESULTS

Identification of related human cells differing in their susceptibility to MVMP and H-1 virus. Two human cell systems were selected for the availability of related cell lines differing in their susceptibility to MVMP, H-1 virus, or both. First, a series of in vitro-transformed or tumor-derived human fibroblasts were compared with nonestablished cultures of corresponding normal cells. In this system, transformation was previously shown to correlate with a concomitant increase in the sensitivity of cells to killing by H-1 virus and MVMP and in their capacity for viral DNA amplification, although virus uptake was not stimulated (7, 9). Then, the established line of human keratinocytes HaCaT (5, 8) was used to isolate a subclone, HaCaT10, that was quite sensitive to H-1 virus-induced killing and a virus-resistant derivative thereof, HaCaT10R2 (Table 1). The close relationship of these two keratinocyte clones was confirmed by showing that all qualitative karyotypic abnormalities of HaCaT10 were also present in HaCaT10R2. These abnormalities consisted of der(2) t(1;2)(q25;q21); t(3q4q); t(6q7p); der(7) t(7;?)(q11,?); i(9q); t(6p;14q); t(4p;18q); ml; m2. Moreover, HaCaT10 and HaCaT10R2 were both essentially triploid (78 to 81 chromosomes) and only differed quantitatively by the presence of four versus three copies of chromosomes 8 and 13 and six versus five copies of chromosome 20, respectively. Thus, the marked difference in the sensitivity of HaCaT10 and HaCaT10R2 cells to H-1 virus (Table 1) may be related to chromosomal imbalances or epigenetic changes, although more subtle qualitative alterations that escaped cytogenetic detection cannot be ruled out. Interestingly, H-1 virus-resistant and -sensitive HaCaT derivatives had a similar growth rate, virus uptake efficiency, and low capacity for viral DNA amplification (Table 1). These clones could not be distinguished by their requirements for epidermal growth factor and insulin when grown in a serum-free medium (data not shown).

Parvoviral protein synthesis. Cultures were infected with 1 to 2 PFU of H-1 virus or MVMP per cell, and de novo viral protein synthesis was measured by metabolic labeling at 18 h p.i. In vitro-transformed (VH10SV, KMST6, SUSM-1, and WI38CT-1) and tumor-derived (HT1080) human fibroblasts supported the synthesis of much greater levels of all four parvoviral polypeptides NS-1, NS-2, VP1, and VP2 (Fig. 1 and 2) compared with corresponding normal cells (VH10 and VH25). This transformation-associated enhancement of cell

FIG. 1. Production of nonstructural (NS-1) and structural (VP1, VP2) proteins of H-1 virus after infection of normal (VH10, VH25) and transformed (VH10SV, KMST6, WI38CT-1, SUSM-1, HT1080) human fibroblasts. ³⁵S-labeled count-matched extracts prepared from infected cultures (2 PFU/cell) at 18 h p.i. were immunoprecipitated with anti-NS-1 (lanes 1 to 7) or anti-VP (lanes 8 to 14) serum and analyzed by gel electrophoresis and fluorography. NS-1*, Hyperphosphorylated form of NS-1. Lane M, Size markers (in kilodaltons).

capacity for viral protein synthesis occurred in most experiments at a similar level for the four polypeptides analyzed, was true for both parvoviruses H-1 and MVMP, and was most pronounced (10- to 30-fold) for the KMST6 line (Fig. 1A, 2A, and 2B). In some experiments, however, the increase in VP1 and VP2 expression was lower than that of the nonstructural proteins. Interestingly, an increase in cell susceptibility to the killing effect of H-1 virus and MVMP was also found to accompany the transformation of human fibroblasts and was the greatest for KMST-6 cells (9), consistent with a causative role of enhanced parvovirus replication in cell sensitization to parvoviral attack.

As in the human fibroblast system, the H-1 virus-sensitive keratinocyte cell line HaCaT10 produced substantially greater levels of all virus-encoded proteins than its resistant

FIG. 2. Synthesis of nonstructural and structural MVMP proteins in a pair of normal (VH25) and transformed (KMST-6) human fibroblasts. Cells were analyzed at 18 h p.i. (2 PFU/cell). (A) ³⁵S-labeled cell extracts were immunoprecipitated with anti-NS-1 (lanes 1 to 4) or anti-VP (lanes 5 to 8) serum (lanes 1, 3, 5, 7, mock-treated cells; lanes 2, 4, 6, 8, MVMP-infected cultures). (B) ³⁵S-labeled extracts from infected (lanes 1 and 2) and mock-treated (lane 3) cells were immunoprecipitated with anti-NS-2 serum; immunoprecipitation of ³²P-labeled extracts from MVMP-infected (lane 4) and mock-infected (lane 5) KMST-6 cells. Lanes M, Size markers (in kilodaltons).

FIG. 3. Expression of nonstructural and structural H-1 virus proteins in infected human keratinocyte lines HaCaT10 and HaCaT10R2. Cells were analyzed at 18 h p.i. (2 PFU/cell). (A) ^{35}S -labeled cell extracts from virus-infected (lanes 2, 4, 6, 8) or mock-treated (lanes 1, 3, 5, 7) cells were immunoprecipitated with anti-NS-1 (lanes 1 to 4) or anti-VP (lanes 5 to 8) serum. (B) Immunoprecipitation of ^{35}S -labeled extracts from HaCaT10R2 (lane 1) and HaCaT10 (lane 2) cells with anti-NS-2 serum. Lanes M, Size markers (in kilodaltons). NS-1*, Hyperphosphorylated form of NS-1.

derivative HaCaT10R2 (Fig. 3). Advantage was taken of the similar growth rates of both keratinocyte clones to compare the expression kinetics of H-1 virus nonstructural proteins after infection of synchronized cell cultures. As shown in Fig. 4, NS-1 and NS-2 proteins were both maximally expressed about 12 h after cell infection and release into S phase. The time courses of NS protein production were similar in HaCaT10 and HaCaT10R2 cultures, indicating that the above-mentioned lower level of viral polypeptides in H-1 virus-resistant versus-sensitive cells can be ascribed to an intrinsically lower proficiency of the former cells rather than a delay in viral protein synthesis. Similarly, the lower capacity of normal than of transformed human fibroblasts for capsid and nonstructural parvoviral protein synthesis was found to be independent of the time postinfection at which the metabolic labeling was performed (data not shown).

FIG. 4. Time course of H-1 virus nonstructural protein synthesis in virus-resistant (HaCaT10R2; $\circ$, $\square$) and -sensitive (HaCaT10; $\bullet$, $\blacksquare$) human keratinocytes (2 PFU/cell). NS-1 (circles) and NS-2 (squares) proteins were immunoprecipitated with specific antisera after metabolic labeling for 1 h at the indicated intervals postinfection. Protein synthesis was measured by densitometric scanning of fluorographs and is expressed in relative absorption units (A).

Processing of parvoviral nonstructural proteins. NS-1 proteins of autonomous parvoviruses have been shown to exist in phosphorylated forms (11, 25, 26, 31, 36). As phosphorylation of proteins plays an important role in their function, it was determined whether related human cell lines differing in their susceptibility to H-1 virus or MVMP achieved various levels of NS-1 protein phosphorylation. NS-1 immunoprecipitates from H-1 virus-infected human fibroblasts (Fig. 1, lanes 1 to 7) and keratinocytes (Fig. 3A, lanes 1 to 4) constituted major species with apparent molecular masses ranging from 76 to 84 kilodaltons (kDa). The additional 92-kDa band was often predominant in the epithelial cells (Fig. 3A) (8, 16) and present to a lesser relative extent in the fibroblasts (Fig. 1). These various protein species could be shifted to a major 76- to 78-kDa band by treatment with acid phosphatase (16; data not shown), indicating that species of higher apparent molecular mass probably represent phosphorylated forms of the NS-1 polypeptide. In the case of MVMP-infected fibroblasts, NS-1 immunoprecipitates constituted two main discrete bands with roughly similar intensities, with molecular masses of 86 to 88 kDa (Fig. 2A, lanes 1 to 4). The slower-migrating protein was previously shown to represent a phosphorylated NS-1 species of MVMP (11). Interestingly, the distribution of NS-1 proteins of H-1 virus and MVMP in these various subspecies was similar among both the fibroblast lines (Fig. 1, lanes 1 to 7; Fig. 2A, lanes 1 to 4) and the keratinocyte clones (Fig. 3A, lanes 1 to 4), irrespective of the cell susceptibilities to virus-induced killing. Thus, cell sensitization to the parvoviruses correlated with a change in the rate of synthesis of NS-1 protein (see above) but apparently not in the modification of this polypeptide by phosphorylation.

NS-2 immunoprecipitates from MVMP-infected (Fig. 2B) and H-1 virus-infected (Fig. 3B) human cells had apparent molecular masses of about 24 and 18 kDa, respectively, in agreement with the deduced amino acid sequences (12, 13). Both proteins contained two major species of generally similar abundancies. The slower-migrating NS-2 species was readily labeled *in vivo* by incubation with $^{32}\text{P}_i$ (Fig. 2B, lane 4), identifying it as a phosphorylated form of NS-2. As in the case of NS-1, the distribution of NS-2 protein between its subspecies was similar in virus-resistant and -sensitive cells, confirming that phosphorylation of nonstructural proteins is unlikely to be a major determinant of the differential susceptibility of the cells studied to H-1 and MVMP viruses.

The NS-1 protein of MVM and H-1 virus was reported to become translocated into the nucleus of permissive human and murine cells (11, 43). Consistently, the normal and transformed human fibroblasts (Fig. 5) and both keratinocyte strains (data not shown) displayed a predominantly nuclear localization of the NS-1 protein when studied by fluorescence microscopy. Moreover, immunoprecipitations of KMS-6 and KMST-6 cell extracts revealed the predominant presence of NS-1 proteins in the nuclear fractions of both cells (data not shown). However, only 1 and 8% of the nuclei from the resistant VH25 and HaCaT10R2 cells, respectively, stained clearly positive for NS-1 (5 PFU/cell). The cells of the sensitive cultures contained approximately 10 (KMST-6) and 2 (HaCaT10) times more positive nuclei than their resistant counterparts. Thus, the elevated synthesis of NS-1 proteins in the sensitive cells seems to be due to both a greater number of cells expressing NS-1 and a higher production per cell. In contrast to the localization of NS-1, immunofluorescence studies revealed a more or less uniform distribution of the NS-2 protein over the cytoplasm and the nucleus of these cells (Fig. 5).

FIG. 5. Localization of the NS proteins in H-1 virus-infected (5 PFU/cell) normal (VH25; panels A and C) and transformed (KMST-6, panels B and D) human fibroblasts. Cells shown in panels A and B were stained with anti-NS-1 serum, and those in panels C and D were stained with anti-NS-2 serum. Bar, 10 μ m.

Intracellular accumulation of parvoviral mRNAs. The steady-state levels of parvoviral mRNAs were determined by Northern blotting (Fig. 6A) and RNase protection (Fig. 6B) experiments. The RNA probe used for the latter assays revealed the major R1, R2, and R3 viral transcripts by generating protected fragments 217, 80, and 65 nucleotides in length, respectively (Fig. 6B). The large intron excised from the P4-driven primary transcript may account for an additional 137-nucleotide protected fragment. According to both methods, MVMP- or H-1 virus-infected susceptible fibroblasts and keratinocytes contained R1, R2, and R3 mRNAs in relative proportions of about 10 to 20, 10 to 25, and 65 to 70%, respectively (Fig. 6A and 7B). A similar distribution was reported for MVMP-infected murine fibroblasts (10, 39). It appears from the comparison of parvovirus-resistant and -sensitive human cells that the former also synthesize all three viral transcripts but at a much lower level than the latter. Thus, the steady-state levels of R1 and R2 mRNAs in MVMP-infected cultures were 10- to 30-fold higher (depending from the experiment) in the transformed KMST-6 line than in normal human fibroblasts, while the R3 transcript was stimulated to a lesser extent (Fig. 6A, lanes 1 and 2; Fig. 6B). A similar 20- to 30-fold-greater abundance of all three H-1 virus transcripts was found in the HaCaT10 clone compared with its resistant counterpart HaCaT10R2 (Fig. 6A, lanes 3 to 6).

Processing of parvoviral transcripts. Although the abundance of H-1 virus mRNAs was much lower in HaCaT10R2 than in HaCaT10 keratinocytes, the resistant clone contained all three major viral transcripts in similar proportions as sensitive cells (Fig. 6A, lanes 3 to 6). Moreover, primary or processed transcripts did not preferentially accumulate in the nucleus of the resistant derivative HaCaT10R2, which displayed the same distribution of viral mRNAs between cytoplasm and nucleus as sensitive HaCaT10 cells, with a major fraction of RNA located in the cytoplasm (Fig. 6A, lanes 3 to 6). Therefore, the differential susceptibility of the human cells studied to parvoviral cytolysis appears to correlate with their relative capacities for accumulating viral

FIG. 6. Abundance of parvoviral transcripts in MVMP- and H-1 virus-infected (2 PFU/cell) human fibroblasts and keratinocytes. Cells were analyzed at 18 h p.i. (A) Steady-state levels of viral mRNAs were determined by Northern blotting of MVMP-infected transformed (lane 1) or normal (lane 2) fibroblasts and of H-1 virus-infected sensitive (lanes 3 and 5) or resistant (lanes 4 and 6) keratinocytes. Total RNAs from pairs of fibroblasts or keratinocytes were matched for their rRNA contents. Total RNA (lanes 1 and 2) or transcripts from equivalent nuclear (lanes 3 and 4) and cytoplasmic (lanes 5 and 6) fractions were analyzed. (B) RNase protection assays were conducted according to the strategy depicted in panel C. By hybridizing to the major viral transcripts (C, three upper lines) or intron (not shown), with the RNA probe, the MVMP-specific part, which is indicated by the heavy segment on the MVMP genome (C, middle line), generates the protected fragments shown by the dotted boxes (C, four bottom lines). Mock-treated (lane 1) and MVMP-infected (lanes 2 to 4) VH25 and KMST-6 cells were compared. Lane 4 is an overexposure of lane 3. TNT, Intron.

transcripts but not with any detectable change in RNA splicing or export from the nucleus.

The difference in parvoviral mRNA concentration between resistant and sensitive cells may be traced back to a variation in either the production or the stability of viral transcripts. With the object of testing the latter possibility, the decay of preexisting H-1 virus mRNAs was measured in infected keratinocytes maintained in the presence of dactinomycin, an inhibitor of de novo RNA synthesis. No significant difference was detected between HaCaT10 and HaCaT10R2 cells with regard to the persistence of individual preformed H-1 virus transcripts (data not shown). Therefore, stability of parvoviral transcripts is unlikely to constitute a major determinant of the differential capacity of the cells analyzed for viral RNA accumulation.

Effect of multiplicity of infection. The data presented in Fig. 1 through 6 were obtained with cultures that had been inoculated with 2 infectious viral units per cell. It was investigated whether the restriction to parvoviral gene expression in resistant cells could be overcome by increasing the multiplicity of infection. This was apparently not the case. Indeed, the rate of synthesis of both the nonstructural polypeptide NS-1 and the capsid proteins VP1 and VP2 was

FIG. 7. Effect of the multiplicity of infection (MOI) on the production of viral proteins and transcripts. (A) Biosynthesis of NS-1 (lanes 1 and 2) and VP1/VP2 (lanes 3 and 4) proteins in VH25 fibroblasts at 18 h p.i. with 2 (lanes 1 and 3) or 50 (lanes 2 and 4) PFU of H-1 virus per cell. (B) Northern blot analysis of cytoplasmic H-1 virus transcripts extracted from HaCaT10R2 keratinocytes at 18 h p.i. with 2 (lane 1) or 50 (lane 2) PFU of H-1 virus per cell. NS-1*, Hyperphosphorylated form of NS-1.

not significantly different between resistant human VH25 fibroblasts infected with either 2 or 50 PFU of H-1 virus per cell (Fig. 7A). The same result was obtained with murine fibroblasts (data not shown). Similarly, increasing the multiplicity of infection from 2 to 50 PFU of H-1 virus per cell had no detectable effect on the steady-state levels of the major viral transcripts in the virus-resistant keratinocyte clone derivative HaCaT10R2 (Fig. 7B). Thus, virus gene expression in virus-resistant cells cannot be increased to the level sustained by their virus-susceptible counterparts solely by increasing the number of infecting viruses. In other words, cellular factors rather than the availability of viral substrates appear to be the limiting factor for the ongoing parvoviral life cycle in the resistant cells analyzed.

DISCUSSION

Differential parvoviral gene expression in virus-resistant and -sensitive human fibroblasts and keratinocytes. In this study, related human cells differing in their susceptibility to MVMP and H-1 virus were compared for their ability to produce viral transcripts and proteins after infection at a low multiplicity. The various parvovirus sensitivities of the human fibroblasts analyzed were associated with their *in vitro* or *in vivo* oncogenic transformation (7, 9), whereas those of the keratinocyte pair under study have not been related so far to other phenotypic alterations. In spite of their different origins and characteristics, both cellular systems displayed a striking correlation between sensitization to parvoviruses and enhanced viral gene expression. Capsid (VP1, VP2) and nonstructural (NS-1, NS-2) viral proteins were all produced at much greater levels by susceptible cells than by corresponding resistant cultures. This stimulation can be accounted for quantitatively by a comparable increase in the steady-state levels of the three major viral transcripts that encode these proteins. In contrast, the processing of residual mRNAs and proteins synthesized in resistant cultures could not be distinguished from that taking place in sensitive lines. Therefore, in the human fibroblast and epithelial cell systems analyzed, the acquisition of sensitivity to H-1 virus and MVMP appears to parallel a major upmodulation of the parvoviral life cycle at the level of or prior to the transcription of the viral genome. The greater capacity of susceptible

lines for parvoviral NS protein synthesis probably contributes to their sensitization to virus-induced killing, since NS polypeptides and *rep* proteins of adeno-associated viruses are suspected of being cytotoxic (6, 23, 35, 42). Yet the accumulation of nonstructural proteins is not the only clue to parvoviral cytopathogenicity. Thus, increasing the multiplicity of infection from 2 to 50 PFU/cell resulted in a drop in cell viability (9, 52), while the production of NS proteins remained virtually unchanged. Whether the input virus particle-mediated cell disturbance, which has been shown to exist for the dependoviruses (54), is responsible for this additional killing at higher input multiplicities remains to be established.

Differential capacity of susceptible fibroblasts and keratinocytes for parvoviral DNA replication. In contrast to their similar proficiencies in parvoviral gene expression, the sensitive human fibroblasts and keratinocytes studied could be distinguished by the capacity of the former and the inefficiency of the latter for H-1 virus DNA amplification. The replication of parvoviral DNA requires virus-encoded nonstructural proteins as well as cellular functions (13). Interestingly, the expression of at least some of the cellular determinants of parvoviral DNA replication appears to be conditional on the physiological state of host cells. Thus, the onset of parvoviral DNA replication relies on a still unidentified S-phase factor(s) (55). Moreover, the present work points to the involvement of an additional cell type-specific function(s). Indeed, proliferating HaCaT10 keratinocytes failed to amplify H-1 virus DNA in spite of their ability to produce similar amounts of NS proteins as transformed human fibroblasts that support viral DNA replication. Such a dissociation of NS protein production from parvoviral DNA amplification is not without precedent, since it was recently reported for some human mammary tumor cell lines (16).

Although virion single-stranded DNA is complementary to viral RNAs, transcriptionally active templates are thought to consist of double-stranded replicative intermediates (13). The keratinocyte clones HaCaT10 and HaCaT10R2 are unlikely to differ significantly with regard to the generation of such intermediates. The resistant and sensitive clones were equally efficient in virus uptake and had similar H-1 virus DNA contents over time. Moreover, in both keratinocyte lines a similar minute fraction of parentally labeled viral DNA became converted to terminally linked duplex forms, as determined by denaturing agarose gel electrophoresis (data not shown). It follows that the much greater abundance of viral mRNAs in the susceptible HaCaT10 line than in its resistant HaCaT10R2 derivative most probably results from an increase in the transcription of viral DNA rather than from the availability of transcription templates. It should be stated, however, that the latter possibility cannot be formally ruled out, since the fraction of input H-1 virus DNA that is converted to replicative forms is too small to allow its reliable quantitative measurement. Yet normal and transformed human fibroblasts could not be distinguished either by the uptake of input virus or the formation of parentally labeled duplex DNA molecules (4, 7, 9). Moreover, another case of enhanced parvoviral gene expression taking place in the absence of detectable stimulation of viral DNA replication was recently reported for certain established lines of rat fibroblasts (10, 52). Although these lines differed from HaCaT cells by their ability to replicate MVMP DNA extensively, they could also be induced to overexpress viral NS-1 protein and become hypersensitive to virus-

induced killing without a concomitant change in the level of MVMP DNA amplification.

Limiting cellular factors controlling parvoviral gene expression. The restriction to H-1 and MVMP virus gene expression in resistant human fibroblasts and keratinocytes could not be overcome by increasing the multiplicity of infection. This finding is in line with a previous report showing that the fractions of undifferentiated embryonic carcinoma cells expressing MVMP capsid proteins was not appreciably enhanced at high multiplicities (30). Thus, the ongoing parvoviral life cycle appears to be controlled by cellular factors that severely limit virus replication in the restrictive cells studied. Based on the foregoing, one may speculate that some of these factors regulate parvoviral DNA transcription in one way or another. Such a possibility was also considered to account for the differential MVM susceptibility of cells belonging to distinct differentiation pathways (3, 18, 47). Thus, a developmentally regulated host cell factor(s) required for MVM transcription was inferred from reciprocal infections of murine fibroblasts and T-lymphoid cell lines by the fibrotropic prototype strain MVMP and the immunosuppressive variant strain MVMI (47). The possible occurrence of a similar type of modulation of the parvoviral life cycle in response to differentiation (47) and transformation, as shown here for human fibroblasts, would be consistent with the dysregulation of differentiation that is known to occur in many transformed cells (2, 34).

The overall stimulation of parvoviral gene expression in susceptible cells could be minimally traced back to the enhanced transcription of the P4-driven unit encoding non-structural polypeptides. Indeed, NS proteins are known to positively *trans*-activate the P38/39 promoter programming the capsid protein genes (15, 41, 42). The physiological state of host cells may modulate factors that positively or negatively control the initiation of transcription from the P4 promoter. Evidence of both types of host cell regulation has been obtained for other viruses (19, 20). This possibility would be in keeping with the fact that cell transformation, shown here to correlate with an enhanced capacity of human fibroblasts for parvoviral gene expression, is accompanied by a change in the availability of *trans*-acting transcription factors (38, 53). Alternatively, the transcription of the NS unit may be stimulated at the elongation level. Parvoviral determinants that interact with the cellular modulators of viral gene transcription remain to be identified. These determinants may consist of upstream or downstream enhancer (or repressing) elements, as in the case of some papovaviruses and retroviruses infecting embryonic carcinoma cells (19). Alternatively, cellular factors may modulate the functioning or intracellular translocation of parvoviral proteins. Experiments are in progress to distinguish between these various possibilities.

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