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Simian virus 40 persistent infection in long-term immortalized human fibroblast cell lines

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Episomal simian virus 40 (SV40) DNA was detected in various SV40-immortalized human fibroblast cell lines, without rearrangements or mutations. In these cells, SV40 established a persistent infection with the release of a viral progeny. However, electron microscopy analysis showed that virions are morphologically altered, whereas infectivity assay indicated that viral production was hampered. The data suggest that in SV40-infected human fibroblasts, some cells support a complete SV40 productive cycle, whereas other cells resist to the SV40 infection. This sort of "balance" observed within the same human fibroblast population may be responsible for the semipermissiveness of these cells to SV40 infection. *Journal of NeuroVirology* (2004) **10,** 250–254.

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Simian virus 40 (SV40) is a neurotropic monkey virus. In the brain of immunosuppressed monkeys, SV40 induces a fatal demyelinating disease, named progressive multifocal leukoencephalopathy (PML) (Holmberg *et al*, 1977; Horvath *et al*, 1992). Recent data support the hypothesis that SV40 is also a human virus. Indeed, the diffusion of SV40 in the human population is documented by the presence of (i) specific SV40-neutralizing antibodies (Abs) in human sera (Basetse *et al*, 2002; Jafar *et al*, 1998), (ii) SV40 T antigen (Tag) Abs in sera of mesothelioma patients (Bright *et al*, 2002), and (iii) SV40 virions in sewage samples (Vastag, 2002). Moreover, SV40 sequences have been detected, although at low DNA viral load, in human brain tumors, other types of human neoplasms, and normal tissues of children and adults (Barbanti-Brodano *et al*, 1998; Lednicky and Butel, 1999; Jasani *et al*, 2001), suggesting that SV40 is able to establish latent/persistent infections in the human host. The mechanism whereby SV40 establishes a persistent infection in human cells is poorly understood. Different human cells can behave as permissive, nonpermissive, or semipermissive for SV40 multiplication, depending on which tissue type they derive from (Bryan and Reddel, 1994). Indeed, human spongioblasts, fetal neural cells, and certain tumor cell lines are lytically infected by SV40 (O'Neill and Carroll, 1981; O'Neill *et al,* 1998) and are considered permissive cells. Human mesothelial cells, on the other hand, do not efficiently support lytic infection, are transformed at a high rate by SV40 (Bocchetta *et al*, 2000), and release SV40 virions as a result of persistent infection (Bocchetta *et al*, 2000; Cacciotti *et al*, 2001). Following SV40 infection, human fibroblasts give rise to a limited viral progeny (Carp and Gilden, 1966; Girardi *et al*, 1966; Koprowski *et al*, 1967; Aaronson and Todaro, 1968) and become transformed after a few passages in culture (Koprowski *et al*, 1962; Pontén *et al*, 1963; Girardi *et al*, 1966); these cells are therefore considered semipermissive. SV40-transformed human fibroblasts maintain a limited life span until they enter a phase of considerable cell death with a consequent decrease in cell number, indicated as "crisis" (Moyer *et al*, 1964; Girardi *et al*, 1965; Huschtscha and Holliday, 1983). The few transformed cells surviving the "crisis" stage display an unlimited life span, and may be cultured as immortal cell lines (Shay and Wright, 1989; Ozer *et al*, 1996). It should

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be noted that the different behavior of precrisis transformed and postcrisis immortalized human fibroblasts was clarified only when new knowledge accumulated in the field. Therefore, the old studies concerning the effect of SV40 infection in human fibroblasts did not always discriminate precrisis from postcrisis immortalized cells (Koprowski *et al*, 1962; Pontén *et al*, 1963; Moyer *et al*, 1964; Girardi *et al*, 1965, 1966).

It has been shown that SV40-transformed human fibroblasts, i.e., still in the precrisis step, carry SV40 DNA in a free episomal form, and release a viral progeny (Girardi *et al*, 1966; Zouzias *et al*, 1980). On the other hand, postcrisis SV40-immortalized human fibroblasts contain viral DNA in an integrated form, and virus production is abolished (Huschtscha and Holliday, 1983; Gish and Botchan, 1987; Hara and Kaji, 1987). Only few studies have reported on free SV40 DNA in postcrisis immortalized human fibroblast cells. In these reports, episomal viral DNAs are always characterized by consistent rearrangements, which preclude the release of a wild-type viral progeny (Maulbecker *et al*, 1992; Huang *et al*, 1999).

Although several studies have investigated SV40 transformation and immortalization of human fibroblasts, the molecular events occurring in these SV40-infected cell lines are not yet completely known. To investigate these processes, we analyzed SV40 at a molecular level in nine different SV40-immortalized human fibroblast cell lines. We studied MRC5-SV1 and MRC5-SV2 cells, derived from MRC-5 human fetal lung fibroblasts infected with two different SV40 strains, DM and 45–54.2, respectively (Huschtscha and Holliday, 1983); WI-26 VA4 and WI-38 VA13 cells from human fetal lung fibroblasts WI-26 and WI-38, infected by SV40 strain 45–54.1; and GM04312, GM04429, GM08207, GM08437, and XP12ROSV from xeroderma pigmentosum skin fibroblasts immortalized by SV40 strain 776 (Coriell Cells Repository). Southern blot hybridizations, with whole SV40 DNA as a probe, revealed the presence of episomal SV40 DNA molecules in the MRC5-SV1, MRC5-SV2, and GM08437 cell lines (Figure 1). Because the presence of episomal viral DNA is not a common feature in SV40-immortalized human fibroblasts (Huschtscha and Holliday, 1983; Gish and Botchan, 1987; Maulbecker *et al*, 1992), we decided to carry out a biological and molecular characterization of the free episomal viral DNA. DNA obtained from MRC5-SV1 and MRC5-SV2 cells was employed in rescue experiments to verify whether a viral progeny can be reactivated. CV-1–permissive monkey cell monolayers were transfected with 5 μg undigested DNA, using the polycation compound Superfect (Qiagen, Milan, Italy) in accordance with the manufacturer's instructions. DNAs from uninfected human fibroblasts WI-38 and from SV40-immortalized WI-26 VA4 were the negative controls, whereas DNA from precrisis WI-38 cells, 3 months after infection with SV40 strain 776,

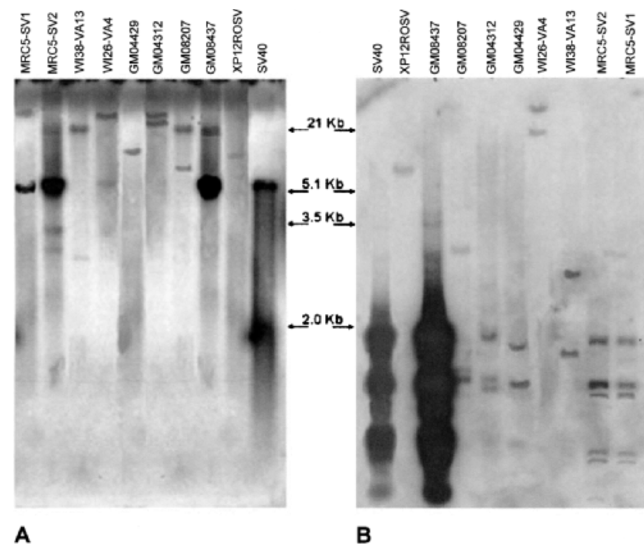

Figure 1 Southern blot analysis of nine SV40-immortalized human fibroblast cell lines, using the whole SV40 DNA as a probe. Ten microgram genomic cell DNA, from samples XP12ROSV-MRC5-SV1, and 3 ng SV40 DNA were digested. (**A**) Analysis of Eco RI–digested samples. A single hybridization band at 5.2 kb, due to linearized SV40 DNA, is present in the SV40 control lane (SV40). A comparable band is present in MRC5-SV1, MRC5-SV2, WI26-VA4, and GM08437 DNA samples. For each human cell line, at least one high-molecular-weight hybridization signal is present, indicating integration of viral DNA into the host genome. (**B**) Analysis of Hin*d* III–digested samples. Six fragments are present in the SV40 control lane (SV40). The same hybridization pattern is displayed by MRC5-SV1, MRC5-SV2, and GM08437 DNA samples, indicating the presence of episomal SV40 DNA. In contrast, only two hybridization signals are present in the WI26-VA4 sample, indicating the absence of free episomal viral DNA.

was used as a positive control. GM08437 cells were excluded from this analysis to avoid any possible false result due to laboratory contaminations. Indeed, GM08437 cells were immortalized with SV40 strain 776, the same as that employed to obtain our positive control. On day 10, CV-1 cells transfected with DNA from MRC5-SV1 and MRC5-SV2 and from precrisis WI-38 cells showed the typical cytopathic effect (CPE) induced by SV40, i.e., vacuoles appearing in the cytoplasm as a pearl neck around the nuclear membrane. On the other hand, no CPE was apparent in the CV-1 cultures transfected with DNA from WI-26 VA4 and WI-38 cells (the negative controls). This result indicates that the SV40 DNA detected in episomal state in the two immortalized cell lines MRC5-SV1 and MRC5-SV2 and in precrisis WI-38 cells was biologically active, whereas the SV40 DNA found integrated in cell line WI-26 VA4 was unable to produce a viral progeny in CV-1–permissive cells.

To verify whether the immortalized fibroblasts MRC5-SV1 and MRC5-SV2 were also able to release a viral progeny, 5×10^5 immortalized fibroblast cells and an equal number of CV-1–permissive cells were plated respectively on the outer and inner chambers of a trans-well apparatus (Corning B. V. Life Science, Schiphol-Rijk, The Netherlands). Precrisis

SV40-infected WI-38 and normal WI-38 fibroblast cells were also cocultured with CV-1 cells as positive and negative controls, respectively. Over 2 weeks, fibroblast monolayers of MRC5-SV1 and MRC5-SV2 and precrisis WI-38 cells grew, whereas CV-1 cells were completely lysed, indicating that human fibroblasts released a viral progeny. On the contrary, CV-1 cells cocultured with the WI-38 normal fibroblasts did not lyse.

To characterize SV40 DNA at a molecular level, viral DNA extraction was performed from virions present in the culture medium of MRC5-SV1 and MRC5-SV2 and precrisis WI-38 fibroblasts. Briefly, 0.2 ml medium was incubated for 1 h at 37°C in the presence of 20 U DNase I, 10 μg RNase A, 25 μl 5% SDS, and 50 mM EDTA, pH 7.5. Samples were kept for 10 min at room temperature, extracted twice with phenol/chloroform and ethanol precipitated. SV40 DNAs recovered from the medium were then fully characterized by polymerase chain reaction (PCR) and filter hybridization with an appropriate internal oligoprobe, as described by Martini *et al* (2002). Restriction-enzyme mapping and DNA sequence analysis of PCR products were performed on whole viral DNA extracted from MRC5-SV2 and precrisis WI-38 cells, for which the complete sequences are available. These experiments confirmed that the viral DNA released by the immortalized cell line and by the precrisis fibroblasts belonged to the SV40 strains used as an input virus (strains 45-54.2 and 776), and did not show any rearrangement or mutation (data not shown).

The presence of SV40 virions in immortalized human fibroblasts was then investigated by electron microscopy (EM) analysis. The viral particles observed in many preparations of the immortalized cells (hereafter indicated as 45–54.2_{MRC5}) were morphologically altered and smaller compared to virions produced in permissive human and monkey cells. In addition, abundant nucleocapsid complexes accumulated in SV40 strain 45–54.2–infected human fibroblasts (Figure 2A). Similar EM images were obtained from human fibroblasts infected with other SV40 strains, such as 776 and DM (data not shown). Normal virions (hereafter indicated as 45–54.$2_{CV\text{-}1}$) were instead detected in CV-1–permissive cells (Figure 2B) infected with the medium from MRC5-SV2 cells, employed as a viral inoculum.

We subsequently investigated whether the altered morphology of 45–54.2_{MRC5} virions could influence virus behavior in relation to the host, by assessing if the same cell receptor was employed by viral particles 45–54.2_{MRC5} and 45–54.$2_{CV\text{-}1}$. Major histocompatibility complex (MHC) class I molecules have been demonstrated to mediate adsorption of SV40 virions on both human and monkey cell surfaces (Breau *et al*, 1992; Atwood and Norkin, 1989). The virion-cell adhesion of 45–54.2_{MRC5} and 45–54.$2_{CV\text{-}1}$ was therefore evaluated after blocking MHC class I receptors on CV-1 cells. These receptors were blocked by incubating CV-1 cells for 45 min at 37°C with 0.3 mg/ml of the specific antibody W6/32, which binds both human and monkey MHC class I molecules (Parham *et al*, 1979; Anderson *et al*, 1998). Cells were then infected, in replica experiments, with 45–54.2_{MRC5} or 45–54.$2_{CV\text{-}1}$, at decreasing multiplicity of infection (M.O.I.); normal CV-1 cells were infected as controls. Twenty-four hours post infection, the number of infected cells in MHC class I blocked and control cultures was determined by immunostaining with the anti-Tag monoclonal antibody Pab 101. The percentage of Tag-expressing cells (i.e., infected cells) in the MHC class I–blocked samples was 40% compared to control cells, for both 45–54.2_{MRC5} and 45–54.$2_{CV\text{-}1}$. These data indicate that, despite their different morphology, 45–54.2_{MRC5} and 45–54.$2_{CV\text{-}1}$ bind the same cellular ligand.

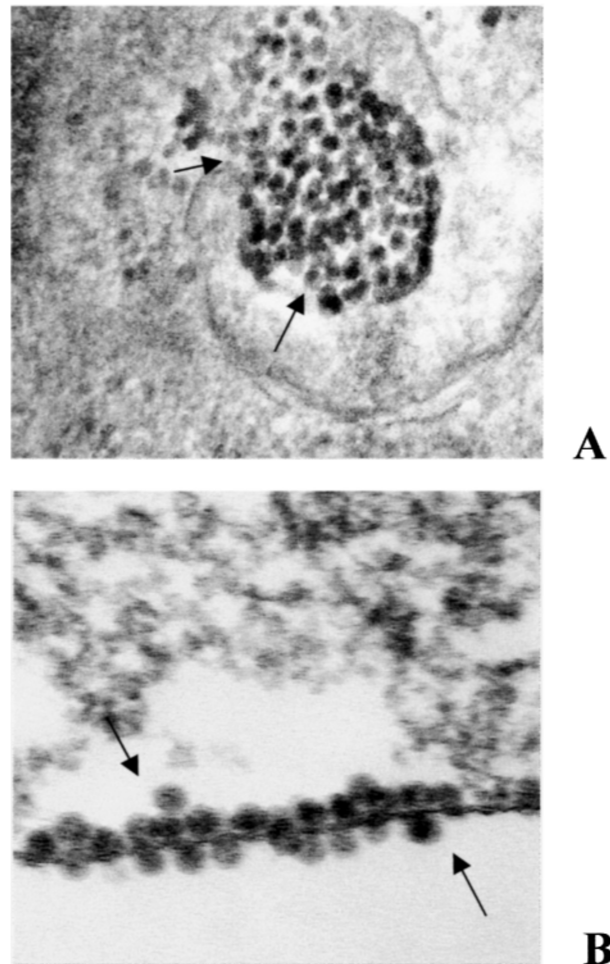

Figure 2 Electron microscopy of human SV40-immortalized MRC5-SV2 fibroblasts (**A**) and CV-1 cells 72 h after SV40 infection with the viral stock previously obtained from MRC5-SV2 cells (45-45-2_{MRC5}) (**B**). (**A**) Morphologically undefined nucleocapsid complexes (*arrows*) are visible in the immortalized human fibroblast MRC5-SV2 sample. (**B**) Several viral particles with a clearly defined morphology are present in the CV-1 cell, mainly adjacent to the cellular membranes (*arrows*). Both images are at a magnification of ×40,000.

In this study, we demonstrate that wild-type SV40 can establish infection in human fibroblasts, persisting even after the "crisis" stage; moreover, an infectious viral progeny is released by long-term immortalized human cells. We found no evidence that molecular rearrangements of the viral genome are required for adaptation to the human host, as suggested by other investigators (Zouzias *et al*, 1980; Gish and Botchan, 1987; Huang *et al*, 1999). Instead, our data imply that a sort of "balance" occurs in the cell population between cells supporting a complete SV40 replicative cycle and those resistant to SV40 infection. The nature of this resistance to infection is unknown. One may speculate that SV40 and human fibroblasts in culture become symbiotic: our hypothesis is that the development of symbiosis is an "active" process, which takes place soon after infection and lasts until the crisis stage. This process results in an effective symbiosis only in a fraction of the cell population, which is the fraction overtaking crisis and able to proliferate indefinitely. In other words, the establishment of the symbiosis occurs in the precrisis cells, and only the few cells where this process is sucessful can recover form the crisis stage and, together with the appearance of specific mutations in the human genome, become immortalized. Our results indicate that SV40 carries out a complete replicative cycle in three (MRC5-SV1, MRC5-SV2, and GM08437) out of the nine cell lines analyzed. At present, it is not clear why SV40 behaves differently in distinct human immortalized cell lines. Probably, some SV40 properties during its life cycle depend on cellular factors rather than on specific SV40 strains or mutants. Indeed, we show by EM analysis that the SV40 virions produced by human fibroblasts display an altered morphology in comparison to that of virions released by CV-1 monkey cells, suggesting that specific interactions with host proteins play an important role in viral particle assembly. The wild-type morphology was instead demonstrated for SV40 virions released by human mesothelial cells (Cacciotti *et al*, 2001). These apparently contradictory results have recently found a possible explanation thanks to the work of Kanesashi *et al* (2003), who demonstrate that the major SV40 capsid protein, VP1, can assemble into structurally different particles depending on environmental conditions. In particular, these authors describe the formation of "tiny particles" (Kanesashi *et al*, 2003) that resemble the viral particles we observed in the SV40-immortalized human fibroblast cell lines.

Because SV40 was accidentally administered to human populations mainly through contaminated polio vaccines during the period 1955 to 1963 (Shah and Nathanson, 1976; Carbone *et al*, 1997), the ability of this viral agent to establish a persistent infection in immortalized human fibroblasts represents a new element to be considered when evaluating its biological activity in the human host. In this perspective, the recent data suggesting that SV40 is now spreading among humans by different routes (Vastag, 2002), independently of the early contaminated vaccines (Barbanti-Brodano *et al*, 1998; Jafar *et al,* 1998; Butel and Lednicky, 1999), acquire a deeper significance. As SV40 adsorbs to monkey and human cell surfaces using the same receptor and is able to multiply in human cells also *in vivo* (Li *et al,* 2002), maintaining the original biological activity, its diffusion in humans seems somewhat more than a hypothesis. Altogether, these data suggest that SV40, known as a monkey virus since 1960, is also a human virus.

The results of this investigation with human fibroblasts, alongside other data obtained with human mesothelial cells (Bocchetta *et al*, 2000; Cacciotti *et al*, 2001), support the notion that SV40 is able to transform, immortalize, and persistently infect human cells, which, in turn, release an infectious viral progeny without lysing. This behavior, displaying characteristics similar to those of RNA tumor viruses, represents a new feature for small DNA tumor viruses such as SV40.

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