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Cytopathic Effects Induced by Epstein-Barr Virus Replication in Epithelial Nasopharyngeal Carcinoma Hybrid Cells

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NPC-KT cl.S61, a subclone derived from an epithelial-nasopharyngeal carcinoma hybrid cell line (NPC-KT), showed cytopathic changes characteristic of herpesvirus replication, including formation of multinucleated giant cells and inclusion bodies, when Epstein-Barr virus replicative cycle was induced by 5-iodo-2′-deoxyuridine. Acyclovir (an inhibitor of herpesvirus DNA polymerase), Epstein-Barr virus-immune human serum, or 2-deoxyglucose (an inhibitor of the glycosylation) interfered with syncytium formation, indicating that a virus-specified glycoprotein belonging to the late group is responsible for cell fusion induced by Epstein-Barr virus replication in cl.S61 cells.

Epstein-Barr virus (EBV) is generally considered to be a lymphotropic virus because of its causative role in infectious mononucleosis, its close association with Burkitt's lymphoma (3, 6), and the ease with which it can transform B lymphocytes in vitro (8). The detection of EBV-associated nuclear antigen (EBNA) and viral DNA in nasopharyngeal carcinoma (NPC) and a few other epithelial carcinomas makes it clear that EBV can infect epithelial tissues and is associated with the transformation of them (19). Sixbey et al. have shown that human epithelial cells explanted from ectocervical tissue and grown as monolayers can be infected with EBV with production of EBNA and early antigen (EA)/capsid antigen (VCA) and with probable viral replication as indicated by in situ cytohybridization (14). Moreover, the detection of viral antigens (including EBNA, EA/VCA, and viral DNA) in epithelial cells of the oropharynx from persons with acute infectious mononucleosis suggests that, in vivo, EBV regularly gains access to and replicates lytically in epithelial cells (13). Epithelial cells of the oropharynx were suggested to be the site of virus replication in vivo (18). Young et al. have shown that two monoclonal antibodies against the CR2/EBV receptor react with pharyngeal epithelial cells in a cell differentiation-dependent manner, raising the possibility of direct virus entry into a naturally exposed epithelium and strengthening the evidence in favor of an epithelial reservoir of EBV infection in vivo (20). In oral hairy leukoplakia in human immunodeficiency virus-infected patients, EBV EA/VCA antigens have been identified by immunofluorescence assays and a high copy number of linear EBV genomes have been detected by Southern blot analysis, indicating that the epithelial cells are permissively infected and contain actively replicating EBV (5). The orally administered acyclovir (ACV) therapy inhibited EBV replication and induced a clinical regression of hairy leukoplakia, indicating that EBV replication produces a lytic infection and hyperplasia of the epithelial cells of the tongue followed by the clinical appearance of oral hairy leukoplakia (9). Thus, EBV replication in epithelial cells produces a lytic infection in vivo; however, viral replication in epithelial cells has not been examined in vitro due to the lack of an appropriate tissue culture system.

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Recently, we have established an EBV-carrying epithelial-NPC hybrid cell line (NPC-KT) by fusing primary NPC epithelial cells with an epithelial cell line derived from human adenoid tissue (15). EBV from NPC-KT cells lacking defective viral DNA molecules has dual properties to transform human B lymphocytes and to superinfect Raji cells, inducing EA and viral DNA synthesis (10–12, 15). By subcloning NPC-KT cells, we obtained a clone designated cl.S61 which produced virus upon induction with 5-iodo-2′-deoxyuridine (IUdR) at higher levels than did the original NPC-KT cells. Moreover, cl.S61 cells producing virus showed cytopathic effects similar to the cytopathic changes which are associated with replication of other herpesvirus, including cell rounding and the formation of multinucleated giant cells, nuclear inclusions, and cytoplasmic inclusion-like bodies. In this communication, we describe the cytopathic changes induced by EBV replication in epithelial cells and discuss the viral function involved in the induction of cell fusion.

An epithelial-NPC hybrid cell line (NPC-KT) was cultured in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum at 34°C. Subcloning of NPC-KT cells was performed at passage 60 by plating 100 cells in culture medium containing 0.3% agarose in a 60-mm plastic dish. Colonies were picked up with a Pasteur pipette 10 days after plating. cl.S61 cells were cultured on glass cover slips for 3 days in the presence of 75 μg of IUdR per ml and for a further 2 days in the absence of the drug. Cells treated or untreated with IUdR were stained with hematoxylin and eosin (Fig. 1), and the number of multinuclear cells was determined (Table 1). cl.S61 cells showed extensive cell destruction at 2 days after IUdR treatment, and multinucleated giant cells, nuclear inclusions, and cytoplasmic inclusion-like bodies were observed frequently in IUdR-treated cells (Fig. 1B and C). More than 80% of cells treated with IUdR were positively stained by indirect immunostaining with serum from a patient with NPC containing antibodies against viral EA and VCA (Fig. 1J). Both nuclear and cytoplasmic inclusions were strongly stained. The original NPC-KT cells did not show rapid cell lysis after IUdR treatment, and neither syncytia nor inclusions were observed in NPC-KT cells expressing EA and VCA (Fig. 1G and H). ACV, an inhibitor of EBV-associated DNA poly-

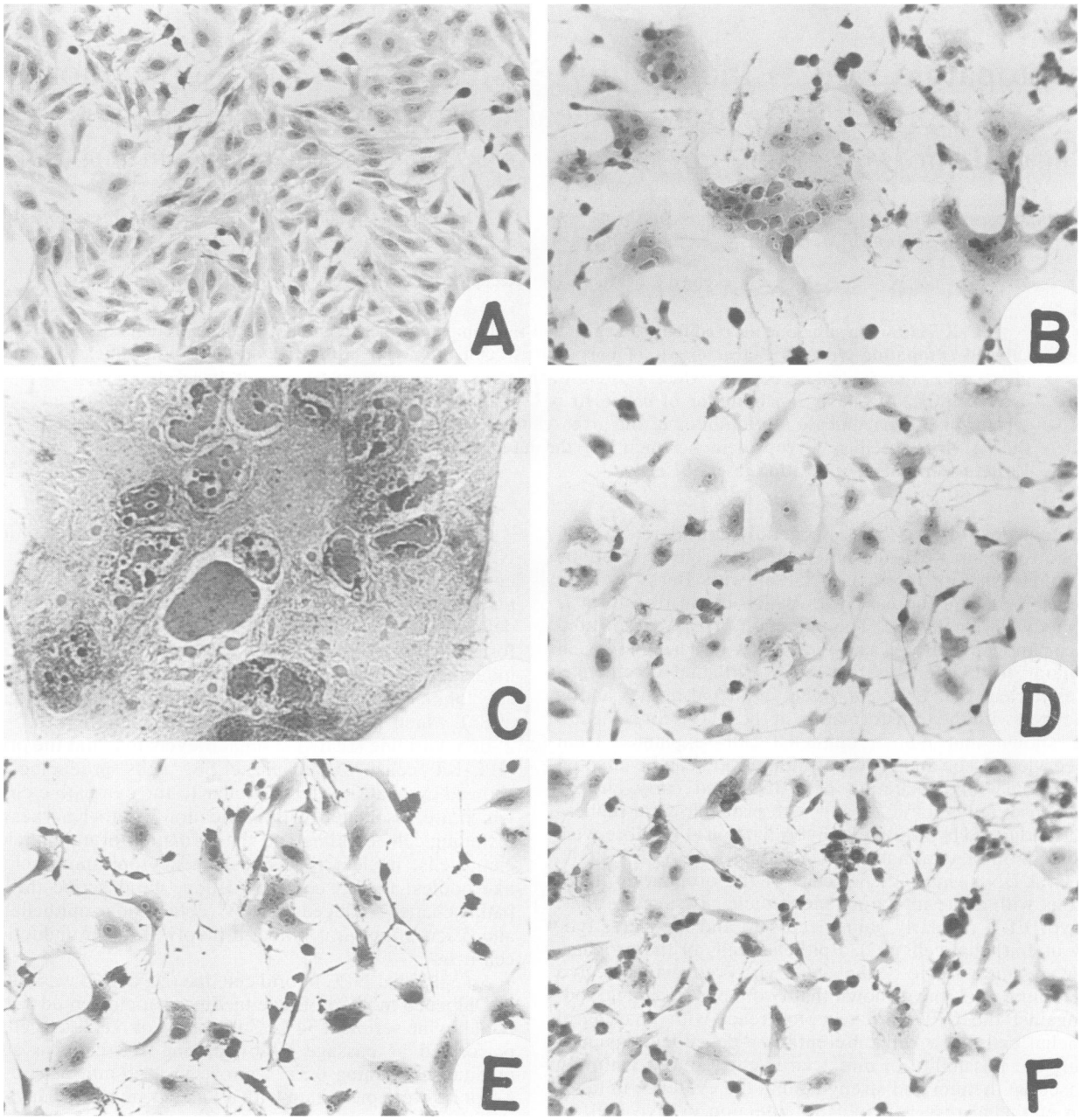

FIG. 1. Induction of cytopathic changes characteristic of herpesvirus replication in cl.S61 cells. Shown are cl.S61 cells either untreated (A), induced by IUdR (B and C), induced in the presence of 100 μM ACV (D), induced in the presence of 5% (vol/vol) heat-inactivated serum from a patient with NPC (E), or induced in the presence of 10 mM 2-deoxyglucose (F). Untreated (G) and IUdR-induced (H) original NPC-KT cells were stained with hematoxylin and eosin. Uninduced (I) and induced (J) cl.S61 cells were also stained with serum from a patient with NPC (EA antibody titer, 1:160; VCA antibody titer, 1:640) by indirect immunofluorescence. (Panels C, I, and J magnifications, ×400; all other magnifications, ×100.)

merase, was included in the culture medium to interfere with the virus replication. The addition of ACV to the culture of cl.S61 cells treated with IUdR inhibited the formation of syncytia and inclusion bodies (Fig. 1D; Table 1). However, cell rounding was not inhibited by ACV. The exposure of IUdR-treated cl.S61 cells to high-titer anti-EBV serum from a patient with NPC also interfered with the syncytium formation (Fig. 1E), but EBV-seronegative serum from a healthy person did not have any significant effect (Table 1). The effect on cell fusion of 2-deoxyglucose, an inhibitor of the glycosylation, was examined. As shown in Fig. 1F and Table 1, the addition of 1 or 10 mM 2-deoxyglucose interfered with syncytium formation. These results indicate that viral glycoprotein expressed on the cell surface at the late stage of viral replication is responsible for cell fusion.

Viral antigens in cl.S61 cells reacted with serum from a patient with NPC or from a seropositive healthy person were compared with those induced in P3HR-1 cells by immunoprecipitation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Viral EA and VCA

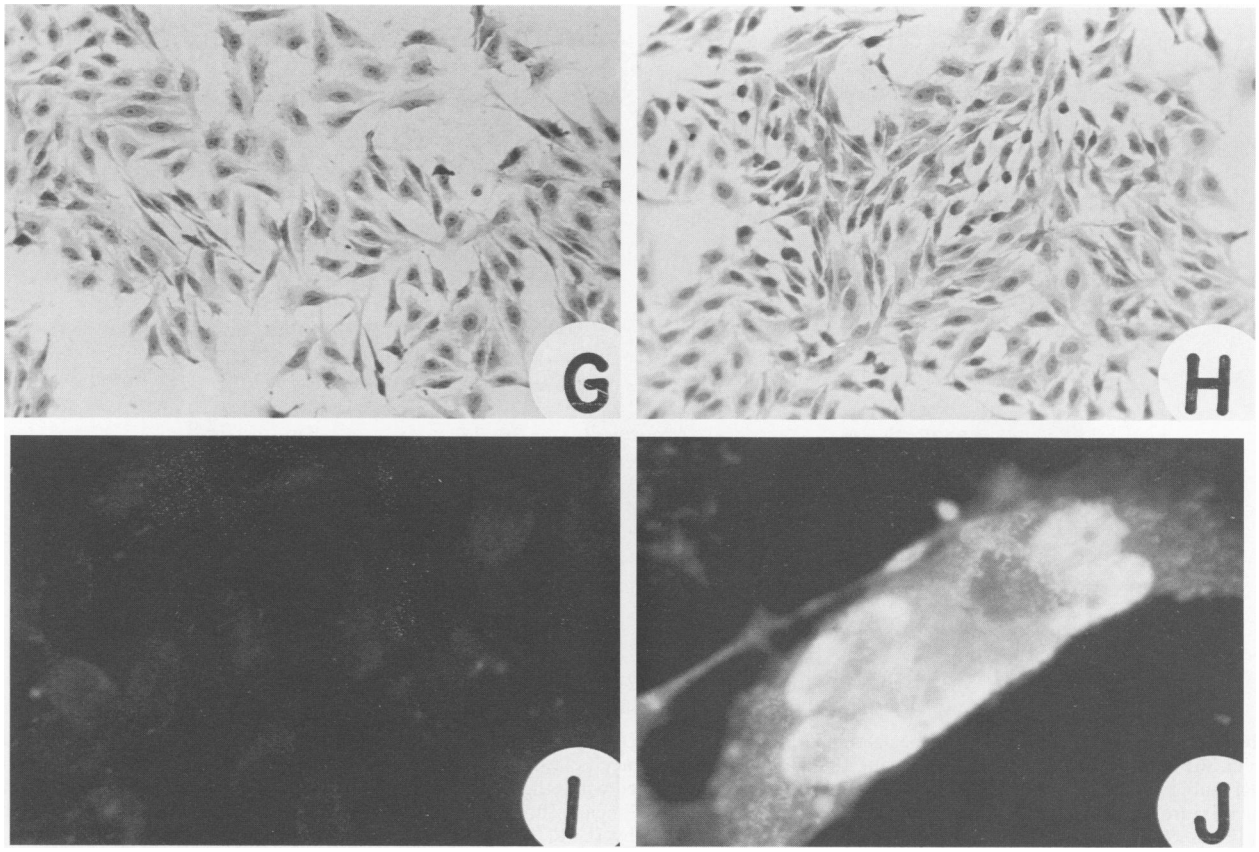

FIG. 1—*Continued.*

polypeptides precipitated with NPC patient serum from cl.S61 cells treated with IUdR were indistinguishable from those from P3HR-1 cells, except for the abundance of a 115-kilodalton (kDa) protein (Fig. 2, lanes 1 and 2). Serum from a seropositive healthy person precipitated VCA polypeptides from cl.S61 and P3HR-1 cells (Fig. 2, lanes 3 and 4). Synthesis of VCA polypeptides was inhibited by the addition of ACV to the culture of induced cl.S61 and P3HR-1 cells; only EA polypeptides were detected with NPC patient serum (lanes 5 and 6), but serum from a seropositive healthy person did not precipitate any detectable bands from ACV-

TABLE 1. Effect of ACV, anti-EBV human serum, and 2-deoxyglucose on the syncytium formation of cl.S61 cells

Culture addition	No. of syncytia/1,000 cells[a]	Inhibition (%)
None	91	
ACV[b]	0	100
EBV-seropositive serum[c]	8	91.2
EBV-seronegative serum[c]	72	20.1
2-Deoxyglucose (1 mM)[d]	5	94.5
2-Deoxyglucose (10 mM)[d]	1	98.9

[a] Number of cells with more than three nuclei per 1,000 cells was counted 2 days after removal of IUdR.

[b] ACV (100 μM) was included in the culture throughout IUdR induction.

[c] Heat-inactivated EBV-seropositive serum from a patient with NPC (EA antibody titer, 1:160; VCA antibody titer, 1:640) (5% [vol/vol]) or seronegative serum from a healthy person (EA antibody titer, <1:5; VCA antibody titer, <1:5) was included in the culture after removal of IUdR.

[d] 2-Deoxyglucose (1 or 10 mM) was included in the culture after removal of IUdR.

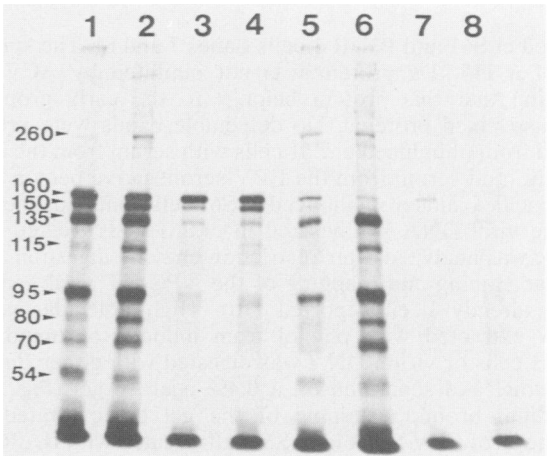

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of EBV-associated polypeptides induced in P3HR-1 and cl.S61 cells by IUdR. P3HR-1 and cl.S61 cells induced by IUdR were labeled with 50 μCi of [^{35}S]methionine per ml for 48 h and immunoprecipitated either with serum from a patient with NPC (EA antibody titer, 1:160; VCA antibody titer, 1:640) (lanes 1 and 2, respectively) or with serum from a seropositive healthy person (EA antibody titer, 1:<5; VCA antibody titer, 1:160) (lanes 3 and 4, respectively) or were labeled in the presence of 100 μM ACV and immunoprecipitated with either NPC patient serum (lanes 5 and 6, respectively) or with serum from a seropositive healthy person (lanes 7 and 8, respectively). Myosin (200 kDa), phosphorylase (92.5 kDa), bovine serum albumin (69 kDa), and ovabumin (46 kDa) labeled with ^{14}C were used as molecular mass markers. Numbers on the left indicate molecular mass in kilodaltons.

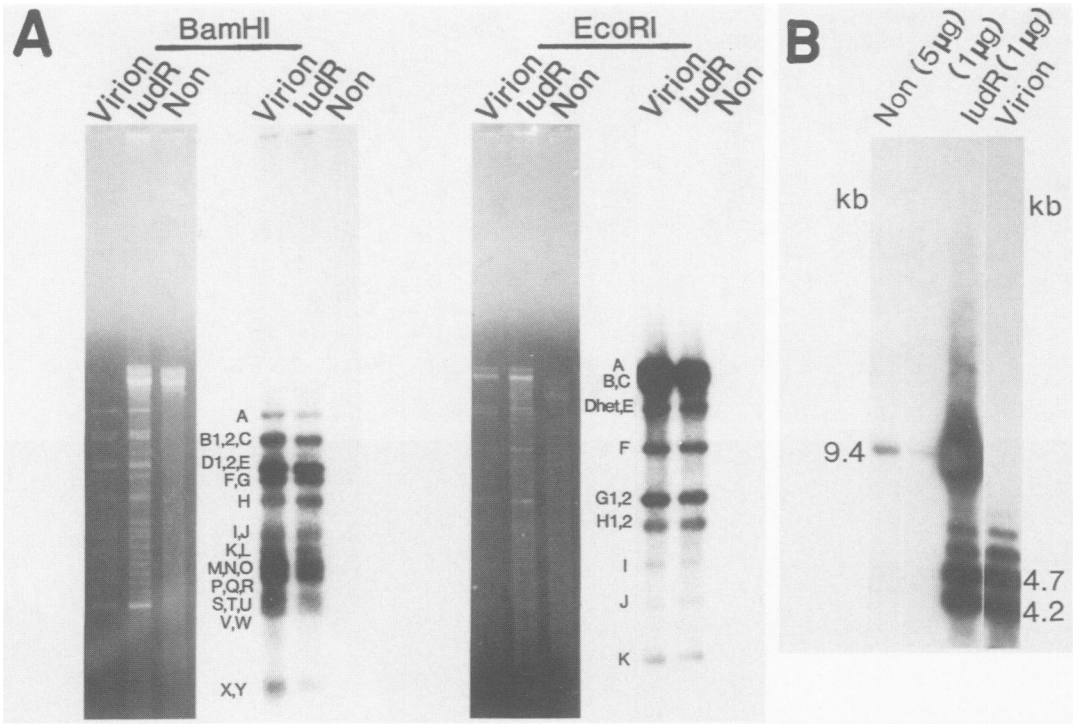

FIG. 3. Analysis of EBV DNA synthesized in cl.S61 cells. (A) DNAs from partially purified virus from cl.S61 cells (0.5 μg; lane Virion), from cl.S61 cells induced by IUdR (5 μg; lane IUdR), and from uninduced cl.S61 cells (5 μg; lane Non) were digested with either *Bam*HI or *Eco*RI, subjected to electrophoresis through a 0.4% agarose gel, stained with ethidium bromide, transferred to nitrocellulose, and hybridized to the mixture of ^{32}P-labeled recombinant EBV DNA cosmid clones (clones 1, 20, 35, and 39) (10). (B) DNAs extracted from uninduced cl.S61 cells [lanes Non (5 μg and 1 μg), respectively], cl.S61 cells induced by IUdR [lane IUdR (1 μg)], and virus from cl.S61 cells (0.5 μg; lane Virion) were digested with *Bam*HI, subjected to electrophoresis through a 0.4% agarose gel, transferred to nitrocellulose, and hybridized to the ^{32}P-labeled *Xho*I 1.9-kb fragment representing unique DNA adjacent to the right-terminal repeats.

treated cl.S61 and P3HR-1 cells (lanes 7 and 8). The synthesis of a 115-kDa protein was not inhibited by ACV, indicating that this protein belongs to the early group of virus-specified proteins. No detectable bands were precipitated from uninduced cl.S61 cells with serum from the NPC patient, and serum from the EBV-seronegative person gave no specific bands from induced cl.S61 cells (data not shown).

The viral DNA synthesized in cl.S61 cells treated with IUdR was analyzed after restriction enzyme digestion. Molecular cloning and mapping of the NPC-KT viral genome have already been reported (10). High-molecular-weight DNA extracted with phenol from induced or uninduced cl.S61 cells or virion DNA was digested with either *Bam*HI or *Eco*RI and separated on a 0.4% agarose gel (Fig. 3A). Ethidium bromide staining of the gel demonstrated the synthesis of EBV DNA in cl.S61 cells treated with IUdR. No detectable viral DNA fragment was observed by ethidium bromide staining in DNA from uninduced cl.S61 cells. After transfer to nitrocellulose, the blots were hybridized to the mixture of ^{32}P-labeled recombinant EBV DNA cosmid clones (clones 1, 20, 39, and 35) which would hybridize to all of the EBV *Bam*HI and *Eco*RI DNA fragments (10). The copy number of viral DNA accumulated in induced cl.S61 cells was estimated from the intensities of viral DNA bands to be more than 3,000 EBV genome equivalents per cell.

In order to examine the terminal structure of EBV DNA synthesized in IUdR-induced cl.S61 cells, Southern blot analysis was performed with the *Xho*I 1.9-kilobase (kb) probe, which represented unique DNA adjacent to the repeat sequence at the right terminus (12). The right-terminal *Bam*HI fragment of EBV DNA contained approximately 3.5 kb of unique DNA and various numbers of 0.5-kb terminal repeats. Since the left *Bam*HI terminus of EBV contains approximately 4 kb of unique DNA and multiple copies of terminal repeats, the fused-terminal fragments, which contained 7.5 kb of unique DNA, could be identified by their larger sizes. The *Xho*I 1.9-kb probe identified a ladder of several fragments of different sizes representing restriction termini of linear forms of virion DNA (Fig. 3B, lane Virion) and a single 9.4-kb fused-terminal fragment in DNA from untreated cl.S61 cells (lane Non). After induction, new DNA forms appeared, including ladder arrays (representative of linear viral DNA) and circular or concatemeric forms (which would be potential replicative intermediates for the synthesis of linear DNA) (Fig. 3B, lane IUdR). These results indicate that EBV actively replicated in cl.S61 cells induced by IUdR.

The data presented here clearly demonstrated that EBV replication in epithelial-NPC hybrid cells, NPC-KT cl.S61, induces cytopathic effects typical of herpesvirus replication (including cell rounding and formation of syncytia and inclusion bodies) and that viral glycoprotein expressed on the cell surface at the late stage of viral replication is responsible for cell fusion. The ability of EBV to cause cell fusion was expected from the significant sequence homology between EBV protein predicted to be encoded by the BALF4 reading frame (gp110) and the herpes simplex virus 1 glycoprotein B, which is required for virus entry into cells and cell fusion (7). gp110 was shown to be a late viral protein (4). However, formation of syncytia and inclusion bodies

was not observed in EBV high-producer lymphoblastoid cell lines, such as B95-8 and P3HR-1. The difference between cl.S61 and lymphoblastoid cell lines might be due to either different viral antigen-expressing abilities or the fact that the formations of syncytia and inclusions are variable, depending on the cell types used for cultivation and the genetic constitution of the virus strain. A 115-kDa protein was overexpressed in induced cl.S61 cells; however, synthesis of this protein was not inhibited by ACV, suggesting that it may not be directly associated with cell fusion. The detection of EBV-induced nuclear inclusions characteristic of herpesvirus replication, in epithelial cells of NPC tumors grown in nude mice, may support the idea that EBV can grow lytically in epithelial cells, resulting in typical cytopathic changes of herpesvirus replication (17).

EBV was reported to fuse Raji cells by superinfection with a strain of P3HR-1 virus (1, 2). EBV from NPC-KT cells also induced EA synthesis and cell fusion by superinfection of Raji cells, and the frequencies of both EA induction and cell fusion were enhanced by the addition of dimethyl sulfoxide to the culture of superinfected Raji cells (11; T. Takimoto, H. Sato, H. Ogura, S. Tanaka, K. Masuda, S. Ishizuka, and R. Umeda, Laryngoscope, in press). However, factors responsible for cell fusion appear to be different between cl.S61 and superinfected Raji cells, because fusion of Raji cells by superinfection with EBV was not inhibited by ACV and the fusion-inducing factor in superinfected Raji cells belongs to the early group of virus-specified proteins (1, 2). Further studies will be necessary to elucidate the mechanism of cell fusion induced by EBV replication, particularly in relation to gp110.

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