Herpesvirus Saimiri-Induced Lymphoblastoid Rabbit Cell Line: Growth Characteristics, Virus Persistence, and Oncogenic Properties

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A nonproducer lymphoblastoid cell line (7710) containing the herpesvirus saimiri (HVS) genome was established from the HVS-positive spleen of a male, inbred New Zealand White rabbit (III/J strain) which had developed a well-differentiated lymphoma after inoculation of HVS and 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Antibodies to HVS early and late antigens were detected in the serum of rabbit 7710 by indirect immunofluorescence and immunoprecipitation. The cell line was of T-cell origin, did not produce HVS, and could not be superinfected with HVS. However, HVS early antigens could be induced in the cells with *n*-butyric acid and TPA or TPA alone. On the other hand, late antigens were never observed, and infectious virus could not be rescued by cocultivation of 7710 cell with OMK cells. The 7710 cells were T-cell growth factor dependent, even after many in vitro passages. The 7710 cell line contained multiple copies of a nonintegrated, covalently closed circular HVS genome. As is characteristic of some other HVS-transformed nonproducer lymphoid cell lines, a large segment of unique light (L) DNA was missing in the persistent circular viral DNA present in 7710 cells. This deletion spanned at least 42.5 kilobases, corresponding to the segment between 12.3 and 50.7 map units of full-length, infectious virion L-DNA. The 7710 cells failed to induce tumors in athymic nude mice, but inbred rabbits inoculated with as few as 100 of these cells developed fatal lymphomas. Chromosomal analysis showed that tumors were due to the growth of donor cells. Cells recovered from one of the rabbits inoculated with 7710 cells also contained HVS DNA and, after in vitro culture, induced the same type of lymphoma when inoculated into two other III/J-strain rabbits. None of the previously described HVS-transformed cell lines have been able to induce tumors in either their host species or nude mice. Thus, our demonstration that the 7710 cell line is readily transplantable in syngeneic rabbits represents the first available model which allows analysis of many biological and molecular aspects of the in vivo oncogenicity of HVS.

Herpesvirus saimiri (HVS), an indigenous virus of squirrel monkeys (*Saimiri sciureus*), causes malignant lymphomas with and without lymphocytic leukemia in several species of New World primates as well as randomly bred and inbred strains of New Zealand White rabbits (2, 3, 5, 6, 12, 13, 15, 25).

HVS can induce malignant lymphoma in the inbred III/J strain of New Zealand White rabbits with greater consistency than in noninbred New Zealand White rabbits (3, 5, 6). We have recently found that the tumor promoter 12-*O*-tetradecanophorbol-13-acetate (TPA) can shorten the incubation period preceding the development of malignant lymphoma in HVS-inoculated III/J rabbits (D. V. Ablashi, unpublished data). We were interested in establishing continuous cell lines from lymphoma-bearing III/J rabbits and investigating their biological and molecular characteristics. It would also be of interest to compare such rabbit cell lines with HVS-induced cell lines established from nonhuman primates.

Various lymphoblastoid cell lines (13-15, 17, 28, 29, 32)

have been established from tumor tissues of marmosets and owl monkeys experimentally infected with HVS or by in vitro infection of peripheral blood mononuclear cells obtained from these nonhuman primates. Many of these cell lines are chronic virus producers, and others become nonproducers after a period of in vitro cultivation ranging from several months to a year or more (13-15, 28, 29). The cells in these cultures are always T lymphocytes (26, 28, 35) and, wherever tested, have displayed natural killer cell-like activity (17).

Molecular studies have shown that nonproducer lines have multiple copies of the HVS genome which are present as nonintegrated, covalently closed circular DNA molecules (18, 36). The DNA of these viral plasmids has been found to be defective in that specific segments present in the unique portion of virion light (L) DNA have been deleted (8, 18, 32). The viral DNA in some nonproducer lines has also been shown to be methylated, which is not the case for the viral DNA of producer cell lines (10).

In this study, we describe the establishment of a cell line designated as line 7710 from the lymphomatous spleen of an HVS-inoculated III/J rabbit. Cells of line 7710 were shown to grow in vitro in the presence of interleukin 2 (IL2, T-cell growth factor). Southern blot analysis revealed a major deletion in the L-DNA of the unique viral DNA. The 7710

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cells were found to transplant readily to strain III/J rabbits, in which they produced malignant T-cell lymphomas, thus providing, for the first time, an *in vivo* model for studying biological and molecular mechanisms of carcinogenesis by HVS.

MATERIALS AND METHODS

Source of rabbit lymphoid cell lines. A male III/J (inbred strain of New Zealand White) rabbit was inoculated intravenously and intraperitoneally with 1 ml (0.5 ml by each route) of partially purified HVS S295C containing $10^{6.5}$ 50% tissue culture infective doses of virus. Concurrently, this rabbit received a single intraperitoneal dose of 500 ng of TPA. This rabbit developed an enlarged spleen and lymph nodes by 22 days after inoculation and died ca. 5 months later. It also developed antibody to HVS antigens as detected by indirect immunofluorescence (IF) and by immunoprecipitation. At necropsy, the spleen, thymus, peripheral lymphocytes, and lymph nodes were taken for cultivation. A diagnosis of malignant lymphoma was made on a subsequent histological examination. Cultures derived from lymphoid tissues were initiated by mincing the tissues and flushing fragments with medium. Released cells were transferred to RPMI 1640 medium supplemented with 20% fetal bovine serum and antibiotics. Peripheral mononuclear cells were prepared by conventional means with lymphocyte separation medium, and after being washed in complete medium, they were seeded at 2×10^6 viable cells per ml. The spleen of rabbit 7710 was the only tissue from which a cell line could be established. Similar attempts to establish lymphoid cell lines from the peripheral blood lymphocytes and lymphoid tissues of several other tumor-bearing, HVS-inoculated rabbits were unsuccessful. Furthermore, rabbits inoculated with TPA alone (500 ng intraperitoneally and 500 ng intravenously) failed to develop discernable disease, and after sacrifice 6 months later, their tissues failed to yield any permanent cell lines.

Animals. Seventeen III/J rabbits, obtained from the Jackson Laboratory, Bar Harbor, Maine, were used for the transplantation of 7710 cells from different *in vitro* passages. The age and sex of these animals are indicated below. The sera of these animals, when tested before inoculation, did not contain antibodies to HVS in the IF assay as described by Klein et al. (20). BALB/c athymic nude mice, 3 to 4 weeks old, were obtained from the Animal Production Unit at the National Cancer Institute Frederick Cancer Research Facility.

Cell culture, virus isolation, and cytogenetic analyses. Rabbit lymphocytes were cultured without prior activation in medium consisting of equal volumes of fresh RPMI 1640 medium supplemented with 20% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 U of penicillin per ml, and 50 μ g of streptomycin per ml; 3-day harvests of medium from MLA144 cells (30) which had been grown in the same medium were filtered through a 0.2- μ m membrane filter. This conditioned medium initially served as a source of IL2 (30), while commercially available purified human IL2 was used at higher passage levels at a final concentrations of 300 μ g/ml.

Virus isolation. A continuous line of OMK (owl monkey kidney) cells was used. An early passage of these cells (7) was obtained from M. Daniel (New England Regional Primate Research Center, Harvard University Medical School, Southborough, Mass.) and maintained in minimal essential medium supplemented with 10% heated fetal calf serum,

L-glutamine, and antibiotics. When monolayers reached 80% confluency, they were maintained in minimal essential medium plus 5% heated fetal calf serum. Virus-producing rabbit lymphocytes were detected in cultured cells by cocultivation, as described below.

IF assay. Immunofluorescence assays to detect HVS early antigens (EA) and late antigens (LA) were carried out as described by Klein et al. (20). Briefly, 7710 cells were washed in phosphate-buffered saline (PBS), deposited in wells of Teflon-coated slides, and air-dried. They were then fixed for 15 min in cold acetone-methanol (50:50), dried, and stored at -70°C until used. For positive controls, HVS-infected OMK cells were grown on slides, washed in PBS, and fixed in the same way as the 7710 cells.

Immunofluorescence was carried out by depositing appropriate dilutions of sera onto individual wells of the Teflon-coated slides. The slides were incubated for 45 min at 37°C in a humidified chamber, washed three times for 10 min each in PBS without Ca^{2+} and Mg^{2+} , and air-dried. Fluorescein isothiocyanate-conjugated anti-human immunoglobulin G (to detect monkey immunoglobulins) or anti-rabbit immunoglobulin G (H and L chains; Cappel Laboratories, Cochranville, Pa.) diluted 1:20 was added, and the slides were incubated for 45 min at 37°C . After being washed as before, the slides were mounted and examined for fluorescence.

To detect EA in 7710 cells, cultures were treated with 20 ng of TPA per ml for 7 days or 20 ng of TPA plus 300 mM *n*-butyric acid for 4 days before fixing the cells (1). As a positive control, HVS-infected OMK cells were maintained in 100 μ g of phosphonoacetic acid per ml until untreated virus-infected OMK cells showed extensive cytopathic effects. The phosphonoacetic acid cultures were then fixed as described above.

Immunoprecipitation. Cultures to be labeled were washed once with PBS and incubated for 30 to 45 min with 8 ml of methionine-free minimal essential medium without serum. After removal of the starvation medium, 5 ml of the same medium containing a 100 $\mu\text{Ci/ml}$ concentration of [^{35}S]methionine was then added to the cultures for 3 h at 37°C . The monolayers were then washed once with PBS and lysed with 1 to 1.5 ml of lysis buffer (0.01 M sodium phosphate [pH 7.2], 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.1 M NaCl, 0.1% NaN_3 , 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 Kallikrein unit of aprotinin per ml). Extracts were clarified by centrifugation for 10 min in a microfuge, antiserum was added to 200- μ l samples which contained ca. 10^7 cpm, and the mixtures were incubated overnight at 4°C . Immunoprecipitates were collected by absorption to protein A-Sepharose and washed three times in lysis buffer. The final pellet was resuspended in 25 μ l of $2\times$ SDS sample buffer (20% glycerol, 2% SDS, 2% β -mercaptoethanol, 0.1 M Tris-hydrochloride [pH 6.8], bromophenol blue) and heated at 100°C for 2 min, and the samples were applied to 7.5 to 15% polyacrylamide slab gels by the discontinuous system of Laemmli (24) and electrophoresed overnight at 50 to 60 V. The gels were enhanced with Enlightening (New England Nuclear Corp., Boston, Mass.), dried, and exposed to Kodak XAR-5 film at -70°C .

Isolation of cellular DNA. A technique based on previously described methods by Kaschka-Dierich et al. (18) and Schirm et al. (32) was used. Approximately 10^8 cells were suspended in 5 ml of PBS and lysed by the addition of 2.5 ml of 3% Sarkosyl in 75 mM Tris-hydrochloride (pH 9.0)–25 mM EDTA. The lysate was incubated for at least 2 h at 37°C

with 1/10 volume of a 0.1% solution of proteinase K preincubated for 1 h at 37°C. After dilution of the lysate with 3 volumes of 50 mM Tris-hydrochloride (pH 8.5), the solution was adjusted with CsCl to a refractive index of 1.4007. DNA gradients were formed by equilibrium centrifugation in a Spinco vertical type VTi 50 rotor for 20 h at 40,000 rpm and 20°C. Fractions were collected, and DNA was localized by reading the optical density at 260 nm of each fraction. The appropriate fractions were pooled and dialyzed versus 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA. DNA was stored at 4°C.

Restriction endonuclease digestion and gel electrophoresis. DNA samples of 3 µg were digested by restriction endonucleases for 2 h, and fragments were electrophoresed in horizontal 0.6% agarose slab gels overnight in TA buffer (40 mM Tris-hydrochloride [pH 7.8], 1 mM EDTA, 2.5 mM sodium acetate). To estimate the number of HVS genomic copies in the rabbit cells, reconstruction experiments with known amounts of cloned restriction fragments of HVS DNA were mixed with 3 µg of OMK DNA and run together. The amount of virion DNA varied between 1.2 and 0.03 ng, corresponding to 20 to 0.5 single-copy genes per cell.

Electrophoretic analysis of HVS DNA. The procedure is based on the method of Gardella et al. (16). Cells (0.2×10^6 to 1.0×10^6) were pelleted in Eppendorf tubes, washed in 1 ml of PBS, and suspended in 50 µl of solution containing 20% Ficoll 400, 1× TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA), 3 µg of RNase per ml, and 0.025% bromophenol blue. The samples were layered on a 0.8% vertical agarose gel at 4°C and overlaid with 100 µl of solution containing 5% Ficoll 400, 1× TBE, 1% SDS, 1 mg of proteinase K per ml, and 0.025% xylene cyanol. The gels were run for 3 h at 10 V and for 12 h at 100 V. Simultaneously, bacterial cells carrying plasmids of various sizes (*Escherichia coli* J53 with pP537 plasmids of 88.7 and 39.8 kilobase pairs [kbp], *Rhizobium meliloti* 30-25 with plasmids of 408 and 195 kbp, and *Agrobacter tumefaciens* C-58 with plasmids of 420 and 200 kbp) were layered onto the gel as described by Eckhardt (11). About 6×10^7 cells were suspended in 25 µl of solution containing 20% Ficoll 400, 1× TBE, 3 µg of RNase per ml, and 0.025% bromophenol blue, and 75 µl of the same buffer containing 1 mg of lysozyme per ml was added. The mixture was incubated for 5 min at room temperature, and then 80 µl was applied to the gel and overlaid with 100 µl of solution containing 5% Ficoll, 1× TBE, and 1% SDS.

Transfer of DNA to nitrocellulose filters and hybridization. Transfer of DNA fragments onto nitrocellulose filters followed the procedure of Southern (33). Cloned viral DNA fragments of HVS strain 11 (21) were labeled by nick translation (31). Hybridization was performed as described by Wahl et al. (34). Filters were incubated at 42°C for 16 to 20 h in a solution which contained 50% formamide, 5× SSC (1× SSC contains 0.15 M NaCl plus 0.015 M sodium citrate), 10% dextran sulfate, 1× Denhardt solution (0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll 400), 100 µg of salmon sperm DNA per ml, and 20 mM sodium phosphate (pH 6.5). Filters were washed at room temperature three times for 5 min each in 2× SSC containing 0.1% SDS and at 55°C for at least 2 h in 0.1× SSC containing 0.1% SDS. After the filters were dried, they were autoradiographed at –80°C on Kodak X-Omat R-film with intensifier screens.

Cytogenetic studies. Karyological analysis of 7710 cells and tissues of inoculated rabbits were performed by trypsin-Giemsa banding as described previously (27).

RESULTS

Establishment of the 7710 cell line. To show that III/J rabbits could be infected with HVS, peripheral blood lymphocytes from rabbits 7710, 7719, and 7720 were periodically cocultivated with OMK cells at different times after being inoculated with HVS. In all cases, the peripheral blood lymphocytes released infectious HVS which was identified by immunofluorescence. These animals were then sacrificed, and the spleen, enlarged lymph nodes, and thymus of all three rabbits were finely minced and placed into culture. Only cells from the spleen of rabbit 7710 proliferated indefinitely and formed cell aggregates (Fig. 1a). Figure 1b illustrates the electron microscopic appearance of tumor cells present in the spleen of rabbit 7710. The DNA from the spleen of rabbit 7710 hybridized to a level of 70% of the value obtained with HVS DNA in a DNA-DNA reassociation assay (4). The reassociation kinetics were essentially the same when DNA from spleen cells at passage 4 was tested (80%). Secondly, passage 7 cells, by in situ hybridization, were uniformly HVS genome positive (data not shown). Although tumor cells from other organs of this animal as well as those from rabbits 7719 and 7720 initially grew in culture, the cultures began to degenerate after passage 3 and could not be maintained. As described below, HVS was consistently observed in these cultures, and it is possible that, in these cultures, the growth of cells was overwhelmed by cell destruction due to viral cytopathicity.

Seroconversion of rabbits inoculated with HVS. All three rabbits inoculated with HVS developed antibodies to EA and LA. Rabbits 7719 and 7720 developed disease within 35 days after being inoculated, but rabbit 7710 did not develop disease until 5 months later. In the latter case, no antibodies could be detected until shortly before death. Antibody titers (determined by immunofluorescence), when first detected 20 days before death, were 1:80 against HVS LA, 1:20 against HVS EA, and 1:10 against HVS membrane antigens. The terminal sera had titers of 1:320, 1:80, and 1:20, respectively. Only later bleedings of rabbit 7710 precipitated HVS antigens present in [³⁵S]methionine-labeled extracts of virus-injected OMK cells (Fig. 2). These same bleedings also precipitated antigens of herpesvirus atelae (HVA) strain 73 present in [³⁵S]methionine-labeled infected cell extracts (lanes 9 and 10), verifying earlier reports (16) which demonstrated by immunofluorescence that the two viruses are closely related. For comparison, antisera to HVA were used to show reciprocal reactivity to HVS antigens present in labeled extracts of OMK cells (lanes 6 and 7).

Biological characterization of the 7710 cell line. The 7710 spleen-derived cells continued to proliferate indefinitely, forming cell aggregates which ranged from a few cells to more than 100 cells (Fig. 1a). Less than 40% of the single cells observed in the culture were viable. Two percent of the cells in the initial culture released HVS when cocultivated with OMK cells. This virus was subsequently identified as strain 295C by restriction enzyme analysis, as described below. When measured by immunofluorescence, ca. 20% of the cells contained HVS LA at passage 1, but only 10% contained HVS LA at the next passage. By passage 4, infectious virus and viral antigens could not be demonstrated in the cells. As was the case for all of the rabbit tissues placed into culture, the cells began to grow poorly at about this time. The smaller clumps began to disappear, and the number of cells in the larger clumps diminished while the viability of the cells dropped to less than 50%.

Since IL2 has been shown to be required for long-term

FIG. 1. (a) Culture of lymphoid cells (passage 20) derived from spleen of rabbit 7710. These cells grow both as large aggregates and as single cells in the presence of IL2, maintaining $\geq 85\%$ viability (unstained; magnification, $\times 56$). (b) Electron micrograph of spleen from rabbit 7710 which died with well-differentiated lymphoma 5 months after inoculation of HVS (magnification, $\times 3,400$). (c) One-micrometer section of Epon-embedded 7710 cells shows cellular and nuclear pleomorphism and mitotic figures (magnification, $\times 600$). (d) Electron micrograph of cultured 7710 cells shows deeply indented and lobulated nuclei with coarse chromatin. Numerous variously sized lysosomes are present in the cytoplasm. One cell presents uropode formation. (magnification, $\times 5,000$). (e) Lung of rabbit 8224 which developed a lymphoma 14 days after inoculation of 7710 cells. Note perivascular infiltrate of poorly differentiated lymphoid cells (magnification, $\times 400$).

growth by many T-cell lines, the medium used to culture the 7710 cell line was supplemented with IL2. The cells immediately began to recover as was evident by an increase in viability to 80 to 90% and in the number of cells in the clumps (50 to 100). The doubling time of the cells was ca. 48 h. Although the cells have been grown continuously for more than 2.5 years, they have remained IL2 dependent.

The 7710 cells from passages 10, 15, and 20 formed T-cell rosettes with sheep erythrocytes and lacked surface immunoglobulins. Although the cells remained negative for virus production and LA expression, HVS EA could be induced in ca. 10% of the cells after treatment with TPA (20 ng/ml) or

with a combination of 20 ng of TPA per ml and 300 mM *n*-butyric acid as previously described (1).

Ultrastructural characteristics. Electron microscopic examination of the spleen from rabbit 7710 indicated that a well-differentiated lymphoma had developed (Fig. 1b). Ultrastructural analysis of the cultured tumor cells indicated considerable cellular and nuclear pleomorphism, and mitotic figures were readily observed. The 7710 cells showed deeply indented and lobulated nuclei with coarse chromatin. Lysosomes of varying sizes and electron density were observed throughout the cytoplasm, and uropode formation was also observed (Fig. 1c and d). The high level of

lysosomes seen in cultured cells (Fig. 1d) is in contrast with the low level seen in the animal spleen (Fig. 1b), whereas in both cases there were abundant golgi and sparse rough endoplasmic reticulum. These ultrastructure features of the 7710 cells are reminiscent of those of HVS-induced T-cell lymphoma lines of nonhuman primate origin (14, 25).

Structure of the HVS genome in 7710 cells. The DNA from 7710 cells from various in vitro passage levels was analyzed by DNA-DNA hybridization as described by Aulakh (4) with a ^3H -labeled HVS DNA probe prepared from HVS strain 11. At all passage levels, the DNA from the cells hybridized to 70 to 80% of the level of homologous HVS DNA.

Previous work utilized electron microscopy and CsCl density gradient centrifugation to show that HVS-transformed simian lymphoid cell lines contain nonintegrated circular viral DNA molecules. In this study, another approach (16) was applied to search for nonintegrated covalently closed DNA circles. Lymphoid cells were lysed within the wells of vertical agarose gels, and the released superhelical DNA was separated from linear chromosomal DNA by electrophoresis. The superhelical viral DNA fraction was visualized by Southern blot hybridization with cloned HVS DNA as a radioactive probe. Figure 3 demonstrates the presence of nonintegrated HVS circular DNA in the 7710 cell line. About 10^6 7710 cells were lysed in parallel with 2×10^5 or 1×10^6 cells of the simian lines 1670 and 70N2, and circular molecules were hybridized with the cloned ^{32}P -labeled *Eco*RI fragment C of HVS strain 11. The 7710 cells contained superhelical viral DNA molecules of a size somewhat larger than 200 kbp. The multiplicity of viral genomes, however, seemed to be significantly lower than in the simian

FIG. 2. Immunoprecipitation of viral antigens. Lanes 1 to 3, [^{35}S]methionine-labeled extract of HVS 295C-infected OMK cells precipitated with 5 μl of sera from rabbit 7710 obtained 25 days before death (lane 1), 11 days later (lane 2), and just before death (lane 3). Lane 4, Detergent-disrupted preparation of [^{35}S]methionine-labeled, purified HVS. Lanes 5 to 7, Immunoprecipitates of HVA 810-infected OMK cells with the same sera used in lanes 1 to 3; lanes 8 to 10, control HVA sera (lane 8 is a negative serum, although positive spider monkey sera were used for lanes 9 and 10) used with the HVA-infected OMK extract.

FIG. 3. Episomal DNA in 7710 cells. (A) Supercoiled DNA was separated from linear DNA by electrophoresis in a 0.8% vertical agarose gel in TBE buffer. Cell line 1670 (lanes a and e [2×10^5]), 7710 cells (lanes b and f [10^6]), and 70N2 cells (lanes c and g [2×10^5 cells] and lanes d and h [10^6 cells]) were applied to the gel and lysed in the slots, followed by separation at high voltage. DNA markers were run on the gel simultaneously. After being photographed in the presence of ethidium bromide, the gel was UV irradiated for 3 min, blotted to nitrocellulose, and hybridized to the ^{32}P -labeled *Eco*RI fragment C of HVS 11 (B).

cell lines 1670 (ca. 210 genome equivalents per cell) and 70N2 (ca. 270 genome equivalents per cell) as reported by Kaschka-Dierich et al. (18).

The number of genome equivalents per 7710 cell was estimated by reconstitution experiments by Southern blot hybridizations and with plasmid and bacteriophage lambda clones representing the entire L-DNA of HVS strain 11 as probes (22). By comparing the intensity of hybridization of *Eco*RI fragments H (Fig. 4A, lanes 3 and 4) and D (Fig. 4A, lanes 1 and 2) to 7710 cellular DNA and to viral DNA levels ranging from 1.2 to 0.03 ng (20 to 0.5 genome equivalents), it was apparent that 7710 cells contained between 10 and 20 HVS genomes per cell. Figure 4A also illustrates the results obtained by hybridizing additional cloned *Eco*RI fragments of HVS strain 11 to 7710 cellular DNA and purified viral DNA. The location of the various probes on the strain 11 restriction endonuclease map is identified in Fig. 4B. Figure 4A summarizes the Southern blot analysis which showed that *Eco*RI fragments A, D, E, and C are fully represented in 7710 cellular DNA, whereas fragments F and B do not hybridize to 7710 cellular DNA. When *Eco*RI fragment G was hybridized to 7710 cellular DNA, a 1.5-kbp fragment was observed (Fig. 5, lane 2), whereas, based on the restriction maps of both HVS strain 11 (used to prepare the cloned probes) and strain SMH1 (indistinguishable from the 295C strain used to induce the 7710 lymphoma [19]), there would have been a 6.8-kbp fragment if total virion DNA had been present. These results suggest that a large deletion in viral L-DNA occurred in the HVS DNA and has persisted in the 7710 nonproducer cell line. To more carefully locate the borders of this deletion, blots of 7710 cellular DNA doubly digested with *Eco*RI and either *Bam*HI or *Bst*EII (Fig. 5, lanes 1 and 3) were probed with *Eco*RI fragment G. The results (Fig. 5) indicate that the right-hand boundary of the deletion is located between the single *Bst*EII and *Bam*HI sites located in *Eco*RI fragment G of strain SMRI (see also

FIG. 4. Mapping the deletion in the HVS DNA present in 7710 cells. (A) Large fragments of the HVS 11 L-DNA were prepared by digestion with *Eco*RI and separation on agarose gels and were labeled with ^{32}P by nick translation. Odd-numbered lanes represent 3 µg of 7710 cellular DNA per lane digested with *Eco*RI; even-numbered lanes represent various amounts of *Eco*RI-digested HVS 11 DNA as follows: lane 2, 1.2 ng; lane 4, 0.6 ng; lane 6, 0.06 ng; lane 8, 0.03 ng; and lanes 10 and 12, 0.6 ng. Each pair of lanes represents hybridization to a different portion of the HVS genome, as indicated by the letter designating the probe used above the lanes and the map of these *Eco*RI fragments in (B).

Fig. 6). By a similar procedure, the left-hand boundary of the deletion in 7710 viral DNA was shown to lie between 4.5 and 12.3 kbp by hybridization of restriction nuclease-digested 7710 DNA to cloned *Kpn*I and *Hind*III fragments (data not shown). Figure 6 summarizes and compares the map positions of restriction enzyme sites determined on both strain 11 and strain SMRI and indicates the structure of the HVS genome present in 7710 cells.

Transplantability of 7710 cells in III/J rabbits and athymic nude mice. To test the oncogenicity of 7710 cells, a total of 14 III/J rabbits was inoculated with various numbers of in vitro-grown cells obtained at passages 4, 5, 12, 15, 18, and 22. The results (Table 1) show that subcutaneously inoculated 7710 cells rapidly induced malignant lymphomas, regardless of the age and sex of the animal. A correlation between the number of cells in the inoculum and survival time was observed, with large inocula killing the rabbits in 12 to 15 days and few cells (≤ 100 viable cells) requiring an average of 35 days. It was difficult to diagnose the onset of disease because the inoculated animals generally appeared physically normal until the day of death. Some of them developed nasal discharge and dyspnea a few hours before death and became anorexic and inactive. Other than a low-grade fever, no significant diagnostic abnormalities were noted. Although some rabbits exhibited varying numbers of atypical cells in their peripheral blood smears (1 to 10%), the number of these cells did not increase rapidly enough to be predictive of tumor burden. The majority of lymph nodes were slightly enlarged, although massive enlargement was never observed. Generally, pulmonary lesions were grossly evident but not severe. The lungs appeared denser than

normal, with some areas suggesting consolidation. The histopathology of these tumors revealed well-differentiated to poorly differentiated lymphomas. Diffuse infiltrates of various organs and tissues with lymphocytic or lymphoblastic cells were quite typical.

All animals had perivascular and peribronchial lymphomatous infiltrates in the lungs (Fig. 1e). This condition was frequently so massive as to appear to be the cause of death. The livers showed periportal infiltration by the lymphomas. The lamina propria of the small intestine was generally involved, with the infiltrate extending into the muscularis. Many other organs were variably infiltrated. In one animal, the muscle bundles of the tongue were separated by the lymphoma, and in several other animals, there were infiltrates in the subcutis (3, 12). Occasionally, areas of necrosis interspersed the lymphomatous infiltrate. The lymph nodes were uniformly affected but not massively so. The follicular architecture was replaced by a monotypic population of poorly or well-differentiated lymphogenous cells; however, in some areas, the marginal sinus was patent and extranodal infiltration was slight. None of the rabbits inoculated with 7710 cells developed antibody to HVS as determined by IF. The cells recovered from the tumors from inoculated rabbits were always male and remained dependent on IL2 for in vitro growth. Cells from these rabbits (e.g., C3600) readily induced lymphoma upon inoculation into new female III/J rabbits (Table 1). HVS could not be isolated by cocultivation of mononuclear cells from the peripheral blood of the inoculated animals nor from cells obtained from the tumors at the site of inoculation. When 15 nude mice were inoculated with several concentrations of 7710 cells (10^5 to 10^7), no

tumors were detected within the 10-week observation period.

Cytogenetic studies. A summary of our cytogenetic studies of 7710 cells and cells recovered from the tumor and lymph node of a female rabbit inoculated with 7710 cells is presented in Table 2. The karyotype of the original 7710 cell line indicated that it was of male origin. Eighty percent of the cells from passage 12 of line 7710 possessed a normal karyotype, and 20% had both numerical and structural abnormalities. The abnormal karyotypes are listed in Table 2. When 7710 cells at passage 12 were inoculated into a female rabbit (8224), the cells from the tumor at the site of inoculation and from an involved lymph node were identified as male (Fig. 7B and C), indicating that these tumor cells were derived from the inoculated cells and not from induction by HVS. Cytogenetic studies of the tumor from a female rabbit (8224) which had received the same passage 12 cells described above (Table 2 and Fig. 7a) showed that 49% of the cells were normal and 51% contained chromosomal abnormalities. The enlarged mesenteric lymph node of the same rabbit (8224) contained 27% karyologically normal tumor cells, whereas 73% of the tumor cells exhibited numerical and structural abnormalities. Analysis of normal and abnormal karyotypes of the mesenteric lymph node of rabbit 8224 by Giemsa trypsin G banding show that chromosome 6 is most frequently involved in abnormalities, followed by chromosome 15. No particular karyological abnormality was invariably present in tumor cells, however, and since the frequency of abnormalities appeared to be increasing rapidly at the passage levels analyzed, it may be necessary to more carefully analyze earlier passage cells to determine whether a specific chromosome lesion is characteristic of this tumor.

DISCUSSION

Until now, there has been no feasible system which permitted testing of HVS-induced tumor cells for their ability to transplant to a new host. The 7710 cell line has two important characteristics which allow testing for transplantability. First, infectious virus is not produced, and second, syngeneic animals are available for cell inoculation. The results with the 7710 rabbit cells indicate that HVS-induced tumor cells are highly transplantable and thus appear to be neoplastically transformed. This conclusion is strengthened by the fact that cells from transplanted tumors remained virus nonproducers, did not induce antiviral antibody in the sera of transplanted rabbits, and retained the male karyotype when inoculated into female rabbits. To our knowledge, this is the first report in which HVS nonproducer cells have been shown to be tumorigenic.

In culture, 7710 cells require supplemental IL2 for sustained viability and growth. However, these same growth factor-dependent cells are able to transplant very efficiently to new hosts. The cells recovered from transplanted tumors display the same IL2 dependence shown by the parental cells, thereby arguing against the selection of IL2-independent cells in vivo. The endogenous level of IL2 present in healthy rabbits may be adequate for these cells. Since corticosteroids inhibit IL2 production, it should be possible to determine whether hormone therapy would lead to an inhibition of tumor growth.

The molecular mechanisms by which the HVS genome can induce and maintain neoplastic transformation are not clear. However, the HVS DNA present in cell lines derived from HVS-induced tumors or after transformation by HVS in vitro (8, 18, 29, 32) typically contains a large deletion in its

FIG. 5. Precise localization of right-hand boundary of the DNA deletion present in the HVS DNA in 7710 cells. *EcoRI* fragment G (Fig. 4B) was used as a probe to measure the size of DNA fragments generated in viral DNA present in 7710 cells after digestion with *EcoRI* alone (lane 2), *BstEII* and *EcoRI* (lane 1), and *BamHI* and *EcoRI* (lane 3). These data indicate that the *BstEII* site at 56.3 kbp is deleted, whereas the nearby *BamHI* site at 57.4 kbp is preserved. The restriction sites can be identified in the map for strain SMHI L DNA (Fig. 6).

unique region, suggesting that much of the viral genome may not be required for maintenance of the transformed state (15). In all cases, both the left and right ends of the L-DNA are preserved. The left-hand L-DNA appears important for transformation and the oncogenic phenotype of HVS. The construction of specific deletion mutants in the left-terminal L region of HVS which are replication competent has been described. Thus, although replication-competent nononcogenic HVS genomes containing deletions in the left-terminal L region have been constructed (9, 23), oncogenicity can be restored to these deletion mutants by reconstitution of the left-terminal L region (R. C. Desrosiers, personal communication).

Daniel et al. (Abstracts of the 3rd International Symposium on Oncogenesis and Herpesviruses, Cambridge, Mass., p. 213, 1977) described four lymphoid cell lines obtained from lymphomas induced in New Zealand white and AC-CRB rabbits with HVA 810. These cell lines contained replication-competent HVS genomes as measured by their ability to produce virus when cocultivated with OMK cells. Induction of tumors in ACCRB rabbits could therefore be attributed to in vivo rescue of HVA. In our experiments, inbred III/J rabbits inoculated with HVS all died of lymphoma. Just before the animal's death, antibodies to HVS could be detected, suggesting that the principal source of immunizing antigen is the tumor cell. The 7710 cell line, derived from the spleen of one of these rabbits, ceased to produce virus when cocultivated with OMK cells, between in vitro passages 4 and 5, and when used to produce tumors in additional rabbits, did not induce virus or antibody in

FIG. 6. Schematic representation of the deletion mapped in the covalently closed circular viral DNA of cell line 7710. The shaded areas depict the regions where the ends of the deletion map, indicating that the precise sites are not known. No fusion product between the left and right boundaries of the deletion could be detected after various digests with restriction endonucleases (*Bam*HI, *Bst*EII, *Eco*RI, *Hind*III, *Kpn*I, and *Sma*I). A mosaic pattern of virion DNA at the left end of L-DNA cannot be excluded. Restriction maps of HVS strains SMHI and 11 are included for comparison.

those animals. This suggests that, during the first few in vitro passages, replication-competent HVS genomes were preferentially shed from the cells, leading to decreased cytopathogenicity and enhanced capacity for long-term growth.

Karyological analysis of 7710 cells and tumor cells obtained by inoculating 7710 cells into additional rabbits revealed that the male karyotype was maintained in female rabbits. This again indicates that the tumor cells are responsible for producing tumors rather than the induction

TABLE 1. Transplantation of 7710 cells in III/J rabbits

Rabbit no.	Sex ^a	Age (months)	Inoculum	No. of cells	In vitro ^b passage no.	Survival (days)	Mean survival (days)	Histopathology ^c	Presence of atypical cells in peripheral blood
7185	F	18	7710 cells	10 ⁸	12	15	13.5	PD	—
7186	M					12		WD	+
8066	F	2	7710 cells	10 ^{7.7}	4	18	17.2	PD	+
8223	F	4			12	16		PD	—
8224	F	4			12	14		PD	—
C3600	F	18			18	19		PD	—
C3601	F	18			18	19		PD	—
C8087	M	5	7710 cells	10 ⁴	15	24	25	PD	—
C8082	F	4			15	26		PD	+
8084	M	5	7710 cells	10 ³	15	24	26	WD	+
B-3	F	3			15	28		PD	+
8085	F	4	7710 cells	10 ²	15	30	35	WD	+
8086	F	2			15	36		PD	+
C7080	F	4			22	39		PD	—
7183	F	18	8224 cells	10 ⁸	5	17	16.5	WD	+
7184	M	18			5	16		WD	+
C7079	F	5	C3600 cells	10 ⁶	5	18		PD	—

^a The 7710 cell line was originally obtained from a male (M) III/J rabbit. To determine whether the inoculum was causing secondary tumors or, alternatively, whether the tumors were being induced by HVS released from 7710 cells, lymph nodes or subcutaneous tumors obtained from female (F) rabbits were cultured briefly in the presence of IL2. Karyological analysis revealed that in all cases the tumor cells were male.

^b Virus was recovered by cocultivation with OMK only up to passage 4.

^c PD, poorly differentiated lymphoma; WD, well-differentiated lymphoma.

FIG. 7. Karyotype of 7710 cells. (A) Male karyotype of cells recovered from female rabbit (8824) tumor induced by 7710 cells; (B and C) Giemsa trypsin G-banded abnormal karyotypes from mesenteric lymph node of rabbit 8224.

TABLE 2. Cytogenetic studies on 7710 cells and on cells from the tumor and lymph node of female rabbit 8224 which was inoculated with 7710 cells^a

Source	No. of chromosomes/metaphase (no. banded)					% With normal karyotype	Abnormal karyotype
	42	43	44	45	46		
7710 cells (in vitro passage 12)	2(1)	1	42(8)	3(1)		80	42, XY, 3p-, +t (6; 15q), -16, -17 45, XY, 12q-, +16
Tumor at site of inoculation (cultured in medium) (rabbit 8224)		7(4)	48(11)			49	43, XY, -3 (2 cells) 43, XY, -4 43, XY, -9 44, XY, -5, +6 44, XY, 9q- 44, XY, 15q- 44, XY, +4, +6, -15, -16
Mesenteric, lymph node (fresh) (rabbit 8224)	1	6(3)	35(5)	1	1	27	42, XY, -19, -20 43, XY, -6 43, XY, -7 43, XY, -11 44, XY, -8, -8, +12, +15 44, XY, +6, -9, -17, + ring 45, XY, -10, +12, +17 46, XY, +5, +6

^a Cytogenetic studies were performed on the 7710 lymphoblastoid cell line, tumor cells (cultured briefly in vitro with IL2), and a freshly obtained mesenteric lymph node from female rabbit 8224 inoculated subcutaneously with the male 7710 line at passage 12.

of a new tumor by HVS released from the 7710 cells. We also observed that at passage level 12, the 7710 cell line appeared to be undergoing a significant level of secondary karyological change, since the 80% of cells at passage 12 that had a normal karyotype shifted to only 27% normal in a metastatic lymph node within a relatively few cell generations (Table 2). Frequently observed chromosomal abnormalities, often involving chromosomes 6 and 15, do not occur at a high enough frequency to be likely sites for protooncogene activation, and no particular karyological feature appears to be a characteristic marker for these cells. The significance of the combined effect of HVS and TPA in the establishment of the cell line is not known.

HVS-induced nonproducer simian cell lines established from tumors or by in vitro transformation have not induced tumors in either nude mice or monkeys. Recently, owl monkey T cells transformed by HVS in vitro failed to induce tumors in the autologous animal (G. Schechter and H. Rabin, unpublished data). Thus, the 7710 cell line, which to our knowledge is the only vivally transformed rabbit cell line derived from an inbred animal in existence, offers significant advantages over HVS-transformed primate cell lines in terms of understanding the biological and molecular aspects of HVS transformation and pathogenesis.

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