of the mammalian *ras* genes to compensate for the gluconeogenic defect of yeast cells lacking RAS^{SC}-2 or the lethality associated with disruption of both yeast RAS^{SC}-1 and yeast RAS^{SC}-2.

We conclude that the capacity of the yeast RAS and mammalian *ras* genes to be biologically active in the heterologous system indicates that their associated functions may also be conserved. The ease with which second-site and pseudorevertants can be obtained in yeast and the facility with which the genes responsible for the reversion can be cloned has opened the way for determining the events in the normal function of the RAS genes. Our results suggest that such data obtained in yeast may have direct relevance to the pathway by which *ras* functions in mammalian cells.

Note added in proof: Kataoka *et al.* have found independently that yeast cells lacking functional RAS^{SC}-1 and RAS^{SC}-2 can remain viable if they carry a mammalian *ras* gene (*24*).

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7 December 1984; accepted 1 February 1985

A Region of the *Herpesvirus saimiri* Genome Required for Oncogenicity

Abstract. Herpesvirus saimiri *naturally infects squirrel monkeys* (Saimiri sciureus) *without producing signs of disease; infection of other New World primates, however, results in a rapidly progressing, malignant, T-cell lymphoma. Results described in this report identify a region of the viral genome that is required for oncogenicity in owl monkeys* (Aotus trivirgatus); *this region is not required for replication of the virus. This is believed to be the first such genomic region identified in a herpesvirus system.*

Unlike other model tumor virus systems, little is known at the molecular level about any herpesvirus-induced oncogenic transformation. The existence of a *Herpesvirus saimiri* variant (11att) that has lost its oncogenic potential provides an opportunity to investigate the basis for the oncogenicity of the parental, oncogenic virus (strain 11) (*1–6*). Previous results demonstrated a deletion of 2.3 kilobase pairs (kbp) from the strain 11 virus genome in the generation of 11att (*2*). Since the 11att variant arose in cell culture, it seemed possible that point mutations or other undetected changes might be responsible for or contribute to the loss of oncogenicity. We wished to determine if the deletion already identified was responsible for the loss of oncogenicity. To address this problem, we constructed replication-competent virus strains with deletions in the same region as the 11att deletion (*3*). In this report we show that the replication-competent deletion mutant S4 is also not oncogenic and that restoring the DNA sequences lost in the S4 and 11att deletion mutants also restores the oncogenicity.

The limits of virion DNA deletions in the S4 and 11att strains of *H. saimiri*, which were described previously, are shown in Fig. 1. The deletion in 11att (2.3 kbp) extends into repetitive H-DNA, leftward from the left H/L-DNA border (*2*). The S4 deletion (4.0 kbp) was constructed by eliminating DNA sequences between two sites cut by the restriction enzyme Sst I (*3*); this deletion includes only L-DNA sequences.

We constructed *H. saimiri* strains in which the sequences lost in the 11att and S4 deletion mutants were replaced. Virion DNA from non-oncogenic deletion mutants was introduced into permissive owl monkey kidney (OMK) cells together with a cloned DNA fragment from strain 11 spanning the deletion. We used the calcium precipitation procedure of Graham and van der Eb (*7*). Cloned DNA's spanning the deletions are indicated in Fig. 1. After viral-induced destruction of the cell monolayer was complete, cell lysates were screened for the presence of individual virus particles that had reacquired the appropriate DNA sequences. Recombinant viruses

that had reacquired the sequences originally deleted were expected to arise through homologous recombination. OMK cells received 0.2 μg of virion DNA plus 2.0 μg of linearized cloned DNA spanning the deletion. This represents a large (>100-fold) molar excess of fragment, resulting in a relatively high percentage of recombinant virus (*3*). Virus obtained from cells that received S4 virion DNA plus pT7.4 DNA contained approximately 10 percent recombinant virus; about 20 percent recombinant virus was obtained from cells that received 11att DNA plus pTP8 DNA. Virion DNA was prepared from selected isolates that appeared positive by colony hybridization. A single homogeneous viral recombinant from each of the transfections was selected for more detailed restriction endonuclease analysis (Fig. 2) and for animal inoculations (Table 1). Analysis of virus resulting from transfection of cells with 11att DNA alone or S4 DNA alone yielded only 11att and S4, as expected. For details of these experiments see Fig. 2.

The single homogeneous viral recombinants from each of the transfections described above were designated 11att-pTP8 and S4-pT7.4. Restriction endonuclease fragmentation patterns of virion DNA from these isolates were compared to those of the parental strain 11 as well as 11att and S4 (Fig. 2). The 4.0-kbp deletion in S4, when compared to 11, was revealed by the absence of the 4.0-kbp Sst I fragment, the shortening of the Taq I 7.4-kbp fragment to 3.4 kbp, the loss of Kpn I + Sma I fragments G and B (1.5 and 10.2 kbp), and the appearance of a new Kpn I + Sma I fragment of 7.7 kbp. The 2.3-kbp deletion in 11att was revealed by the loss of Sst I fragments of 4.0 and 0.45 kbp and the appearance of a new Sst I fragment of 3.5 kbp (containing H-DNA), the loss of the Taq I 7.4-kbp fragment and the appearance of a new Taq I fragment of 6.0 kbp (containing H-DNA), and the loss of Kpn I + Sma I fragment G (Fig. 2) [for further details see (*2*, *3*)]. The 11att-pTP8 and S4-pT7.4 recombinant viruses have regained the sequences lost in the 11att and S4 deletion mutants such that 11att-pTP8 and S4-pT7.4 have the same restriction endonuclease fragmentation profiles as the parental strain 11 (Fig. 2).

For the experiments in vivo we used owl monkeys (*Aotus trivirgatus*). As controls, two owl monkeys received the parental strain 11 virus and one received strain 11att. As expected, the animals inoculated with strain 11 died after 27 and 33 days (Table 1). This is consistent with previous results with *H. saimiri* strain 11 (*8*). Also as expected, the animal inoculated with strain 11att remained alive and healthy (Table 1). Two of two owl monkeys also survived inoculation with strain S4 (Table 1). Owl monkeys receiving strains 11att and S4 became persistently infected, since we were able to recover virus from peripheral lymphocytes long after experimental infection. We were first able to recover virus from inoculated animals 7 to 21 days after inoculation. Animals inoculated with 11att-pTP8 and S4-pT7.4 died 21 to 31 days after inoculation. Thus, restoration of the DNA sequences restored the pathogenicity of the virus.

Table 1. Pathogenicity of *Herpesvirus saimiri* strains in owl monkeys. Owl monkeys were inoculated intramuscularly with 10^5 infectious virus particles of a cell-free lysate in the right lateral thigh.

Animal number	Virus strain	Infection*	Survival (days)	Pathology
149-80	11	+	27	Lymphoma
452-76	11	+	33	Lymphoma
561-74	11att	+	>200†	
156-80	S4	+	>200†	
157-80	S4	+	>200†	
119-81	11att-pTP8	+	21	Lymphoma
402-74	11att-pTP8	+	27	Lymphoma
416-78	S4-pT7.4	+	24	Lymphoma
502-80	S4-pT7.4	+	31	Lymphoma

*Infection evidenced by recovery of virus from 10^6 peripheral lymphocytes beginning 7 to 21 days after inoculation. Virus was recovered by cocultivation with owl monkey kidney cells. †Owl monkeys were still alive >200 days after inoculation.

The two animals inoculated with *H. saimiri* strain 11 and the four animals inoculated with the two recombinant *H. saimiri* strains, that is, two with 11att-pTP8 and two with S4-pT7.4, died between 21 and 33 days after inoculation with microscopic evidence of malignant lymphoma. Two of the six had hematologic evidence of leukemia with immature lymphoblasts in their peripheral blood prior to death.

Histologically, the lymph nodes of all

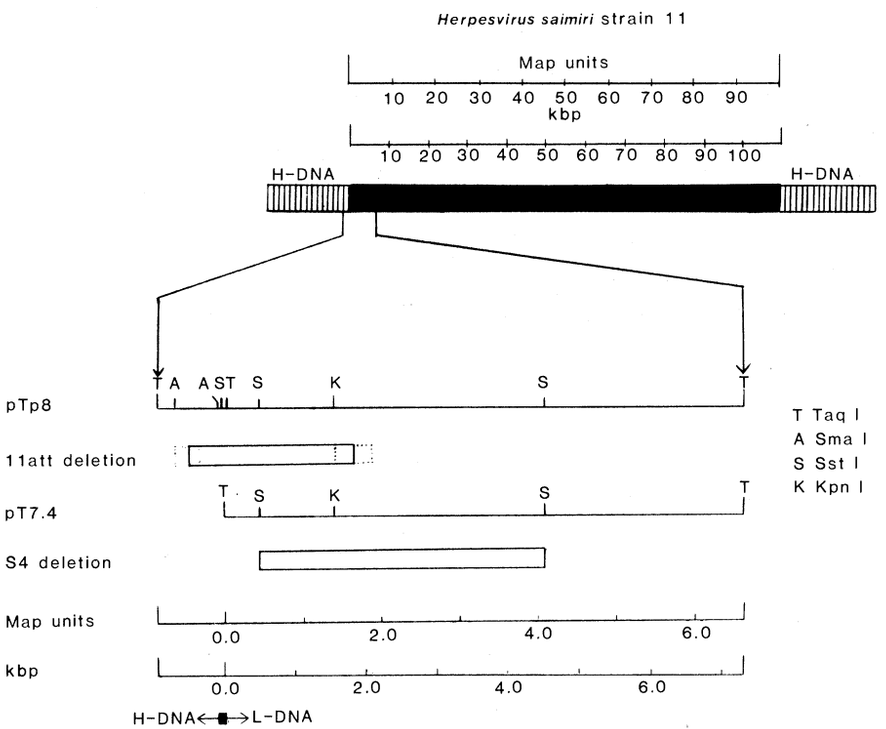
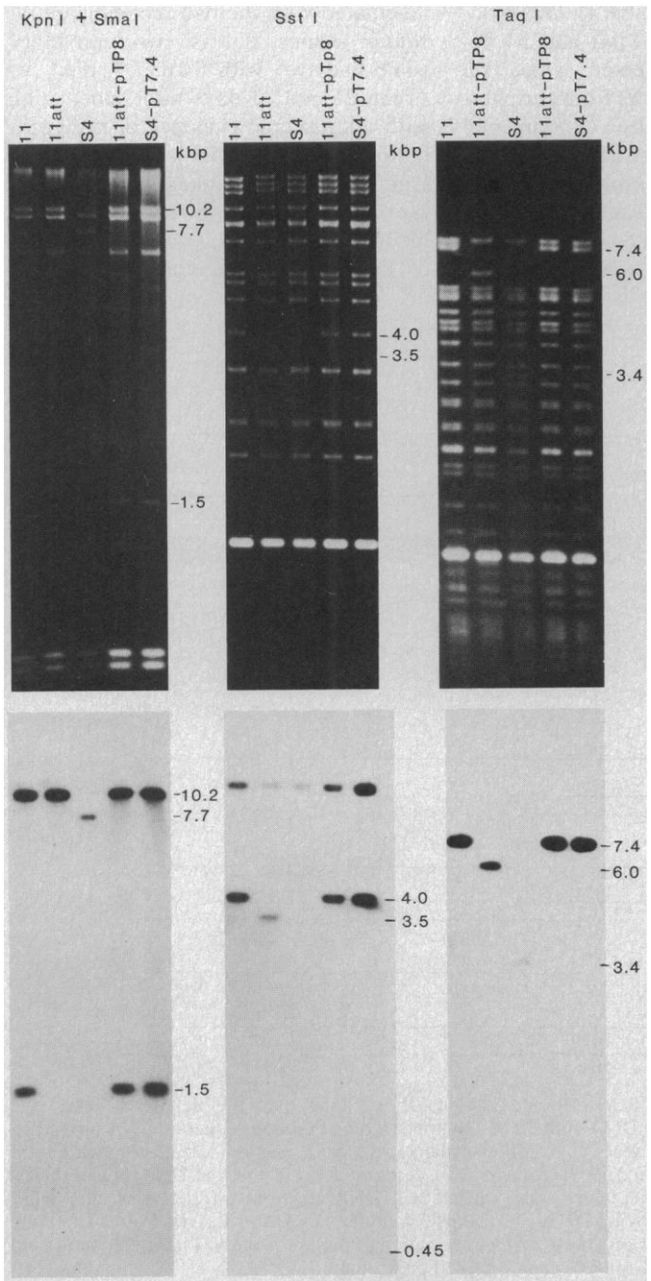


Fig. 1. Location of the deletions and cloned DNA's that span the deletions. Infectious *Herpesvirus saimiri* virion DNA contains a 110-kbp stretch of unique sequence DNA termed L-DNA (light, 36 percent, in G + C content) flanked at each end by a variable number of repetitive DNA units termed H-DNA (heavy, 71 percent G + C). Each H-DNA repeat unit is 1.4 kbp. Cloned DNA pTP8, which spans the left H-L-DNA junction, was derived from a Taq I partial digest of strain 11 virion DNA (*2*). The pT7.4 cloned DNA was derived from a complete Taq I digest of strain 11 virion DNA (*3*). Determination of the limits of the 11att deletion (*2*) and of the constructed S4 deletion (*3*) was described previously.

six animals contained diffuse infiltrates of neoplastic lymphoblasts that obliterated the normal follicular architecture. The tumor cells were somewhat variable in size and had a small rim of eosinophilic cytoplasm, round to ovoid nuclei with a reticular chromatin pattern, and a single prominent nucleolus. Mitotic figures were present in moderate numbers among the neoplastic infiltrates. The capsule of the affected nodes was invaded in only two of the six animals. The spleens of all six animals appeared depleted of the normal lymphoid follicles and periarteriolar lymphoid sheaths, but did contain many neoplastic lymphoblastoid cells throughout the red pulp. A characteristic feature in all animals was the invasion of the walls of splenic veins by tumor cells (Fig. 3, a and b). Other organs invaded by tumor cells in some, but not all, animals were the adrenals, liver, and bone marrow. The one owl monkey that received strain 11att was killed 203 days after inoculation. Histologic examination revealed no evidence of lymphoproliferative abnormalities.

Total cellular DNA was prepared from brain, lymph node, and spleen of owl monkey 119-81 and from brain and lymph node of owl monkey 402-74, both of whom received strain 11att-pTP8. DNA was also prepared from owl monkey 149-80 during the terminal, leukemic phase of disease. The presence of viral DNA was analyzed by Southern blot hybridization with ^{32}P-labeled pT7.4 DNA (data not shown). If one assumes 6×10^6 kbp per diploid cell, the spleen of owl monkey 119-81 contained approximately three viral genome copies per cell while the lymph node of this same animal contained less than one viral genome copy per cell. This difference in viral genome copy number is consistent with the histopathology that showed a greater involvement of the spleen in this animal. The lymph node of monkey 402-74 also contained about three genome copies per cell while the peripheral lymphocytes of 149-80 contained about 30 viral genome copies per cell. Significant amounts of viral DNA were not detected



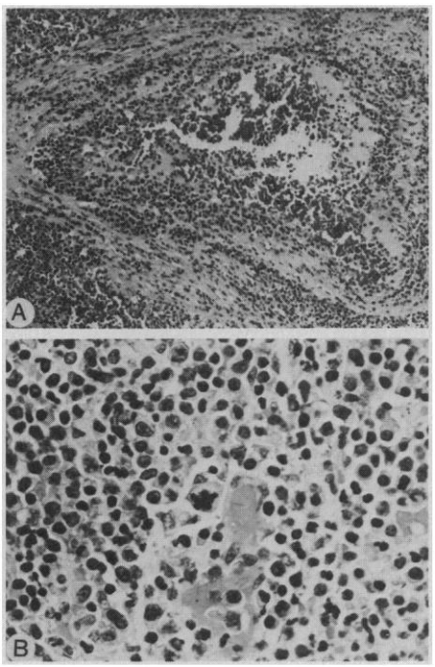


Fig. 2 (left). Isolation and characterization of recombinant virus. Infectious virion DNA (0.2 μg of 11att or S4) was introduced into OMK cells, by the calcium precipitation procedure of Graham and van der Eb (*7*), together with linearized cloned DNA (2.0 μg of pTP8 or pT7.4). After complete destruction of the monolayer (14 days), virus was diluted (6×10^{-7} dilution) in complete minimum essential medium and 150 μl was added to individual wells of OMK cells growing in 96-well Linbro trays. Twenty-three percent (11att plus pTP8) and 16 percent (S4 plus pT7.4) of wells yielded virus, which corresponds, respectively, to a multiplicity of infection of 0.26 and 0.17 infectious virus particles per well (*3*). Portions (2 μl) from virus positive wells were spotted onto nitrocellulose and the DNA was denatured in situ and hybridized with a ^{32}P-labeled DNA fragment from within the deleted region. ^{32}P-labeled pHp1.4 (0.1 to 1.4 map units) was used to detect 11att-pTP8 recombinants and ^{32}P-labeled 4.0 kbp Sst I fragment (0.4 to 4.0 map units) was used to detect S4-pT7.4 recombinants. After rinsing, the filter was exposed to film. Virus stocks were made from wells positive in this colony hybridization assay. Preliminary analysis of small-scale virion DNA preparations was used to select a single homogeneous recombinant from each transfection for more detailed analysis. Virion DNA was prepared and analyzed by agarose gel electrophoresis as described (*3*). Top is ethidium bromide stain and bottom is Southern transfer (*10*) hybridization to a ^{32}P-labeled 7.4-kbp fragment of pT7.4. Fig. 3 (right). Histopathology of the lymphoma. (A) Photomicrograph of a hematoxylin- and eosin-stained, 5-μm section of spleen from *Aotus trivirgatus* 402-74 that had been injected with the recombinant *Herpesvirus saimiri* 11att-pTP8 27 days earlier. The wall of a large splenic vein has been extensively invaded by neoplastic lymphoid cells (×100). (B) Higher magnification of tumor cells in the same spleen as in (A). The invading cells are pleomorphic, have varying amounts of cytoplasm, and contain nuclei that vary from leptochromatic with a prominent nucleolus to those that are very hyperchromatic. An abnormal mitotic figure is present in the center of the field (×400).

in brain tissue. These results confirmed the presence of viral DNA in affected organs.

These observations clearly demonstrate the importance of this region of the viral genome for the oncogenicity of the virus. The deletion mutants described here have allowed us to identify a region of the *H. saimiri* genome required for expression of the lymphoma-inducing capacity of the virus. This region of the genome is apparently not necessary for replication of the virus; the deletion mutants grow as well as parental virus in cultured OMK and Vero cells. Also, the deletion mutant strains are able to infect New World primates, since virus can be recovered repeatedly after experimental inoculation. However, it is not known at what stage these deletion mutants are defective for oncogenic transformation. Studies of the ability of these *H. saimiri* strains and cloned DNA fragments to function in a variety of transformation systems in vitro may help us to understand the mechanisms underlying the oncogenicity of this virus. Recent results have shown that in permissively infected OMK cells, one minor and two major polyadenylated RNA's are affected by the S4 and 11att deletions (*9*). However, these polyadenylated RNA's were not detected in tumor cell lines or tumor biopsy samples. The only RNA detected in tumor cells from the leftmost 7.4 kbp of L-DNA was a 100-base-pair RNA; this 100-base-pair RNA was not detected in permissively infected cells. The role of these gene products in oncogenic transformation by *H. saimiri* remains to be investigated.

Note added in proof: We have recently developed a highly reproducible assay for in vitro immortalization of New World primate T-lymphocytes with *H. saimiri* strain 11. The S4 and 11att deletion mutants do not immortalize in this assay system.

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11. Supported by PHS grants 31363 (R.C.D.) and 26225 (L.A.F.) awarded by the National Cancer Institute, by a special fellowship from the Leukemia Society of America (R.C.D.) and by grant RR00168 to the New England Regional Primate Research Center from the Division of Research Resources, National Institutes of Health. We thank R. Byington for laboratory assistance.
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1 October 1984; accepted 3 December 1984

Hypomethylation of DNA from Benign and Malignant Human Colon Neoplasms

Abstract. *The methylation state of DNA from human colon tissue displaying neoplastic growth was determined by means of restriction endonuclease analysis. When compared to DNA from adjacent normal tissue, DNA from both benign colon polyps and malignant carcinomas was substantially hypomethylated. With the use of probes for growth hormone, γ-globin, α-chorionic gonadotropin, and γ-crystallin, methylation changes were detected in all 23 neoplastic growths examined. Benign polyps were hypomethylated to a degree similar to that in malignant tissue. These results indicate that hypomethylation is a consistent biochemical characteristic of human colonic tumors and is an alteration in the DNA that precedes malignancy.*

Changes in the genome have long been proposed to play a role in abnormal cell growth (*1*). Genomic abnormalities including rearrangements (*2*), mutations (*3*), amplifications (*4*), deletions (*5*), and alterations in methylation (*6*) have all been reported in neoplastic cells. Some or all of these aberrations may result in abnormal gene expression. Two crucial questions from both a mechanistic and a clinical standpoint are when, during the process of neoplasia, can a particular change in DNA structure first be detected? and how often does this change occur in tumors of a given type? In this report, these questions were addressed with regard to alterations in DNA methylation by comparing DNA from a large number of benign and malignant human colon neoplasms with DNA from adjacent, normal colonic tissue obtained from the same patients.

DNA methylation is a covalent modification of the genome that occurs in many

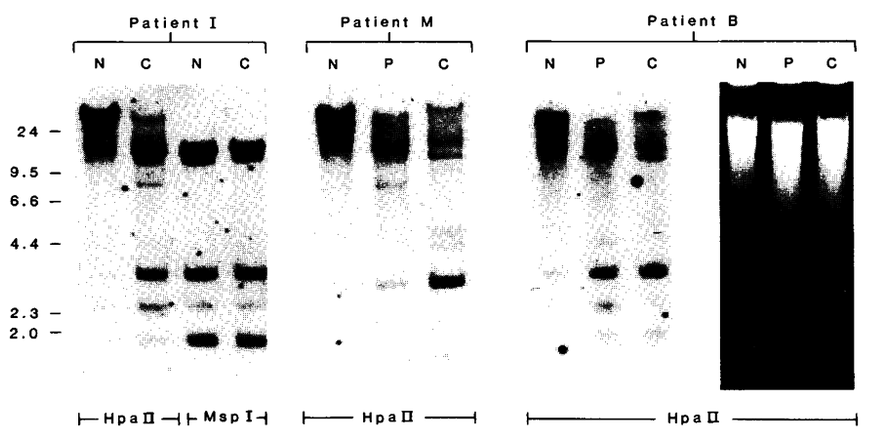

Fig. 1. Analysis of the methylation state of the crystallin gene in normal and in neoplastic colonic tissue. DNA was prepared (*23*) and each sample (5 μg) was digested with Hpa II, Msp I, or Hha I (each at 5 U/μg) at 37°C for 12 to 16 hours. The samples were fractionated by electrophoresis on 1.0 percent agarose gels, stained with ethidium bromide (*24*), and photographed. The gels were then washed (*23*) and the DNA transferred to nitrocellulose essentially as described (*25*). Prehybridizations and hybridizations (12 to 20 hours) were performed (*24*), and the filters were washed as described (*26*) except that the first three washes were for 30 minutes and the fourth and fifth washes were in 0.3× standard saline citrate and 3 percent sodium dodecyl sulfate (for 45 minutes each). Autoradiography was for 16 hours with intensifying screens. Since the Msp I patterns were identical for all 23 neoplasms studied, the Msp I pattern from only one patient is displayed. Molecular weight markers were bacteriophage λ-DNA digested with Hind III. N, normal; P, benign polyp; C, malignant cancer.