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Viral-encoded small RNAs in herpes virus saimiri induced tumors

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DNA sequences from the left terminus of herpes virus saimiri L-DNA are essential for the oncogenic and transforming potential of the virus, but these sequences are not required for replication. RNA derived from 0.0 to 6.7 map units (7.4 kbp) on the herpes virus saimiri genome was studied by Northern blot hybridization and by nuclease protection analyses. Although several poly(A)-containing RNAs were detected from this region in permissively-infected monolayer cells *in vitro*, these RNAs could not be detected in cells taken directly from viral-induced lymphomas nor in the lymphoblastoid tumor cell line 1670. Instead, these transformed T-cells expressed four small RNAs of approximately 73, 105, 110 and 135 nt derived from this region. These small RNAs were not detected at all during the course of lytic infection of monolayer cells. Thus, synthesis of these RNAs is stringently regulated in a cell-type specific manner. Genomic coding sequences for each of these small RNAs were mapped to 0.5-1.2 kbp DNA fragments stretched over 4.3 kbp of viral genetic information. These findings together with the biological properties of mutants with deletions in this region have led us to speculate that one or more of these small RNAs play an essential role in cell growth transformation by herpes virus saimiri.

Key words: small RNA/herpes virus saimiri/lymphoma/transformation

Introduction

Herpes virus saimiri is a lymphotropic herpes virus of New World primates. Immortalized T-cell lines can be established following *in vitro* infection by the virus (Falk *et al.*, 1983; Schirm *et al.*, 1984; Rabin *et al.*, 1984; Desrosiers *et al.*, 1985) and experimental infection of several species of New World primates results in a rapidly progressive malignant T-cell lymphoma (for review see Fleckenstein and Desrosiers, 1982). Little information is available on the mechanisms of oncogenic transformation by this or any other herpes virus.

Although not required for replication of the virus, sequences between 0.4 and 4.0 map units (see Figure 1) are required for transformation and oncogenicity. Two mutants (11att and S4) with deletions in this region have lost their transforming and oncogenic capacity; restoration of the deleted sequences restored these capacities (Desrosiers *et al.*, 1985; Desrosiers *et al.*, 1986). These mutant strains grow as well as wild-type parental virus in permissive monolayer cells *in vitro*, are still able to infect New World primates, retain their ability to persistently infect lymphoid cells *in vivo* and *in vitro*, and are indistinguishable from the parental virus by a variety of other criteria (Schaffer *et al.*, 1975; Wright, 1978; Desrosiers *et al.*, 1985). The function of these viral DNA sequences in transformation is the focus of our current investigations.

Here we report that in tumor cells the only RNAs detectable from the left-most 7.4 kbp of L-DNA are four small RNAs; the genomic coding sequences for two of these small RNAs fall within the sequences previously shown to be required for transformation and oncogenicity.

Results

Herpes virus saimiri contains a 110-kbp stretch of unique sequence DNA called L-DNA (light, 36% G+C). This is flanked at each end by a variable number of 1.4-kbp H-DNA repeat units (heavy, 71% G+C) (Figure 1). We have examined the RNAs derived from the left-most 7.4 kbp of L-DNA in lymphoma tissue samples as well as in owl monkey kidney (OMK) cells lytically infected with strain 11.

Total cell RNA was prepared from samples of liver, spleen and lymph node from an owl monkey inoculated with strain 11 virus; histological examination revealed microscopic evidence of malignant lymphoma in all of these tissues. In addition, RNA was prepared from peripheral blood lymphocytes, since the inoculated animal showed hematological evidence of leukemia with immature lymphoblasts in its peripheral blood. RNA was also prepared from uninfected OMK cells and from OMK cells lytically infected *in vitro* with herpes virus saimiri strain 11. RNA samples were electrophoresed through agarose gels, transferred to nitrocellulose and then sequentially hybridized to ^{32}P-labeled DNA subclones (pHP1.4, pHp3.1 and pHp2.5) derived from pT7.4 (Figure 1). When RNA on the Northern blot was hybridized with ^{32}P-labeled pHp1.4 DNA, two major RNAs, of 4.9 kb and 2.3 kb, were detected in strain 11 infected OMK cells along with a minor RNA of 6.5 kb (Figure 2A). The 5′ and 3′ ends of these RNAs as well as their exon-intron topography have been described previously (Kamine *et al.*, 1984). However, these RNAs were not detected in any of the lymphoma samples and no other transcripts could be detected in the tissue samples with this probe; detectable signal was not obtained with the pHp1.4 probe even after loading 5-10 μg of poly(A)-containing RNA from the lymphoma samples (data not shown). When this blot was stripped of ^{32}P-labeled pHp1.4 and the bound RNA rehybridized to ^{32}P-labeled pHp3.1, small RNA of ~100 nt in length was detected in the lymphoma samples from liver, spleen and lymph node as well as in the RNA from peripheral lymphocytes of the inoculated owl monkey (Figure 2B). This small RNA was absent from both uninfected OMK cells and OMK cells lytically infected with herpes virus saimiari strain 11. In the case of lytically infected OMK cells, only the four major RNAs of 6.5 kb, 4.9 kb, 2.3 kb and 1.4 kb reported previously (Kamine *et al.*, 1984) were detected with this probe. The lymph node sample contained the most and the liver sample the least amount of small RNA; this result was consistent with the histological

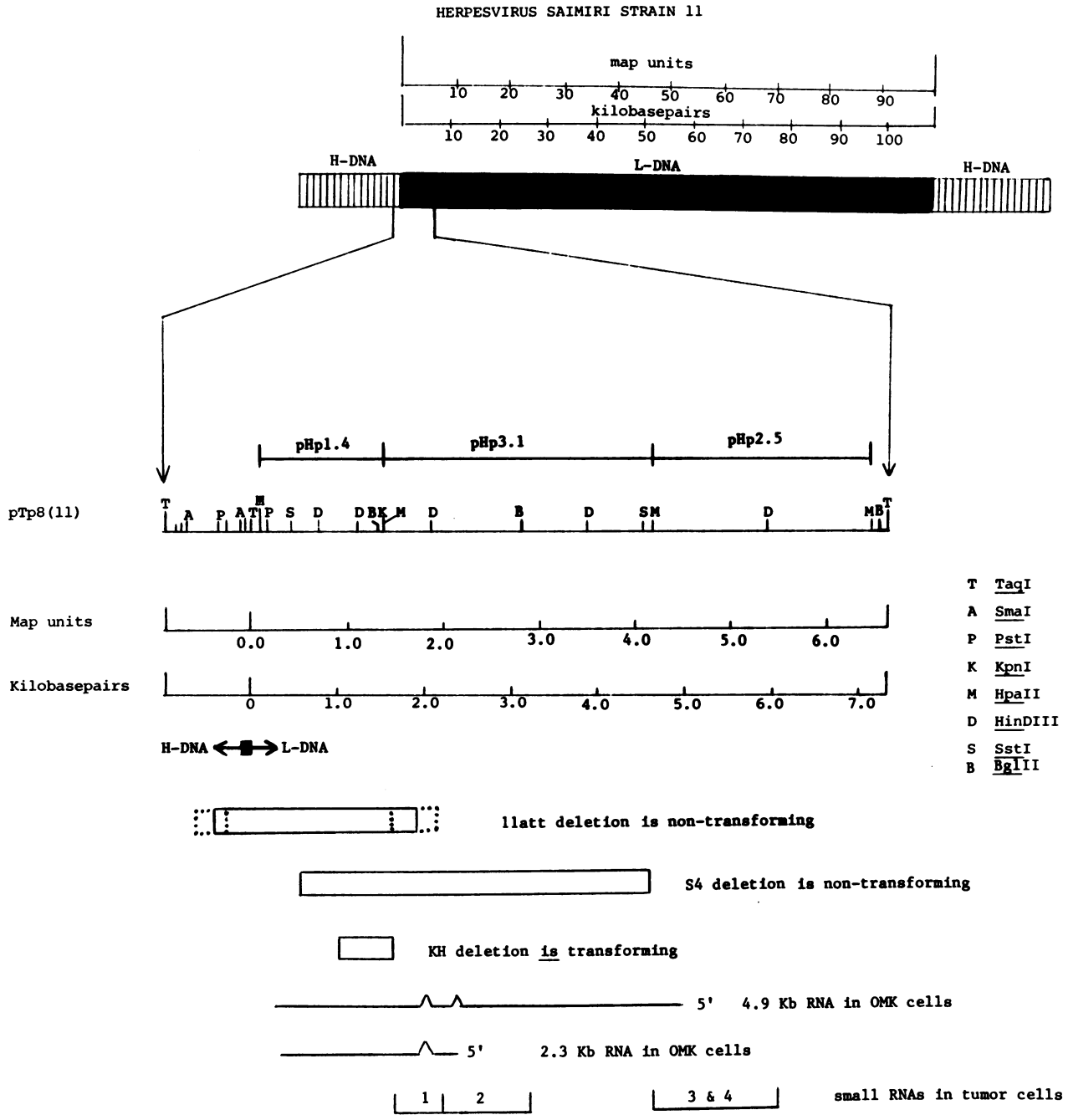

Fig. 1. Summary of information on the region of the herpes virus saimiri genome required for oncogenicity. Derivation of the molecular clones pT7.4, pTP8, pHp1.4, pHp3.1 and pHp2.5 from the left end of L-DNA have been described previously (Desrosiers *et al.*, 1984; Koomey *et al.*, 1984). pT7.4 is a molecular clone of a 7.4-kbp *Taq*I fragment derived from herpes virus saimiri sequences from 0.0 to 6.7 map units. Construction of the S4 and KH deletion mutants and their transforming and oncogenic potentials have also been published (Desrosiers *et al.*, 1984, 1985, 1986); description of the spontaneous deletion in 11att has also been described previously (Koomey *et al.*, 1984). Mapping of the 2.3- and 4.9-kb transcripts in lytically infected OMK cells was accomplished by S1 nuclease and exonuclease VII mapping procedures (Kamine *et al.*, 1984). The small RNAs 1, 2, 3 and 4 refer to the RNAs of 135, 110, 105 and 73 nt found in tumor cells; the vertical lines before and after the numbered small RNAs indicate the restriction sites within which each small RNA maps.

analysis of these tissues which showed more extensive infiltration of neoplastic lymphoblasts in lymph nodes and spleen than in the liver of the inoculated animal. The blot was stripped of radiolabeled pHp3.1 DNA and the RNA was hybridized to radiolabeled pHp2.5 DNA, the *Hpa*II subclone derived from the right end of pT7.4 (Figure 2C). Again, the RNA from liver, spleen and lymph node as well as the RNA from peripheral lymphocytes contained small RNA that hybridized to the pHp2.5 probe. This RNA was not detected in uninfected OMK cells. The OMK cells infected with strain 11 virus contained only the five high mol. wt RNAs reported previously and appeared to completely lack the small RNA observed in the tissue samples. On this Northern blot (Figure 2A, B and C), RNA from strain 11 infected OMK cells was extracted from the cells at 3 days after the cells had been infected with virus, at a multiplicity of infection of one. However, we have also infected OMK cells with strain 11 virus at a multiplicity of infection of 10 and were unable to detect the small RNA at any time point during the course of lytic infection

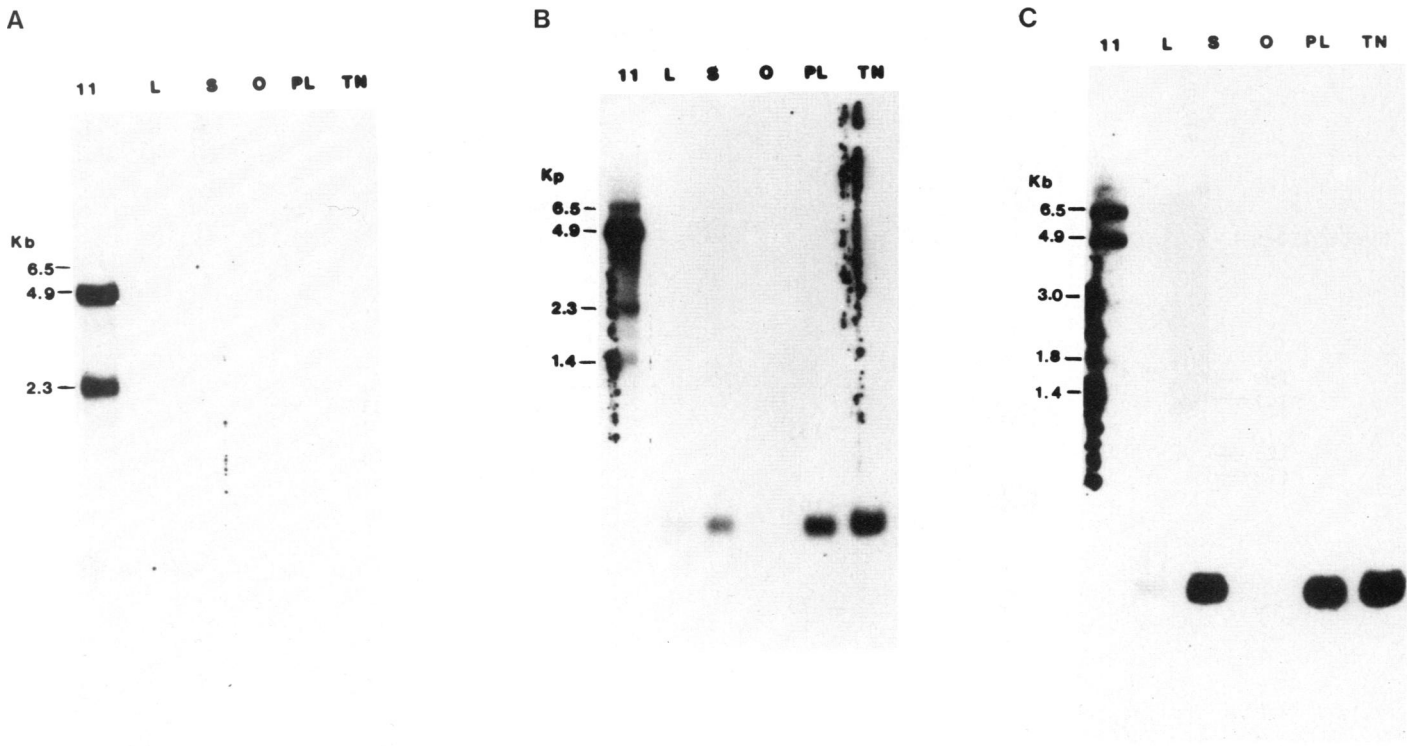

Fig. 2. Northern blot analysis of RNAs from cell lines and tissue samples. 10^7 d.p.m. of each probe was used: **(A)** pHp1.4, **(B)** pHp3.1, **(C)** pHp2.5. Each slot contained 6 μg of RNA. 11, OMK cells infected with strain 11 virus; L, lymphoma, liver; S, lymphoma, spleen; TN, lymphoma, lymph node; O, uninfected OMK cells; PL, peripheral lymphocytes from leukemic stage. L, S, TN and PL were from same owl monkey following infection with herpes virus saimiri strain 11.

(data not shown). In a separate Northern blot of these same RNA samples, when the order of the sequential hybridization with these probes was reversed, the pHp2.5 and pHp3.1 probes again were able to detect small lymphoma RNA while the pHp1.4 probe gave a negative result. In other experiments, small lymphoma RNA did not bind to oligodeoxythymidylate–cellulose and we believe it to be non-polyadenylated. It is RNA by virtue of its complete sensitivity to alkali (0.05 N NaOH, 20 min at 37°C) (data not shown). We have also detected the small RNA in preparations from tissue samples of four other owl monkeys inoculated with strain 11 virus and similarly have not detected the larger poly(A)-containing RNAs in these four additional lymphoma samples.

Small RNA was also detected in the 1670 tumor cell line (Figure 3). This tumor cell line was established from a herpes virus saimiri-induced marmoset lymphoma. When transferred RNA was hybridized with ^{32}P-labeled pHp2.5 DNA, RNA of ~100 nt was detected in total cellular RNA prepared from 1670 cells but not from molt-3 cells (a human T-cell line) and not from the Epstein–Barr virus immortalized owl monkey B-cell line, VII. Brain tissue from the lymphomatous owl monkey was negative for this RNA as were lymph nodes from an uninfected owl monkey (Figure 3).

Since agarose gels would not resolve multiple low mol. wt RNA species, RNA from 1670 cells was electrophoresed through 8% polyacrylamide–8 M urea gels, electroblotted to nylon membranes and hybridized with ^{32}P-labeled, cloned DNA probes (Figure 4). Radiolabeled pHp3.1 DNA hybridized with two RNA species of ~135 and 110 nt, and labeled pHp2.5 hybridized with two additional RNAs of ~105 and 73 nt. Thus, the small RNA from 1670 cells shown in Figure 3 can be resolved into four separate RNA species by electrophoresis through polyacrylamide gels.

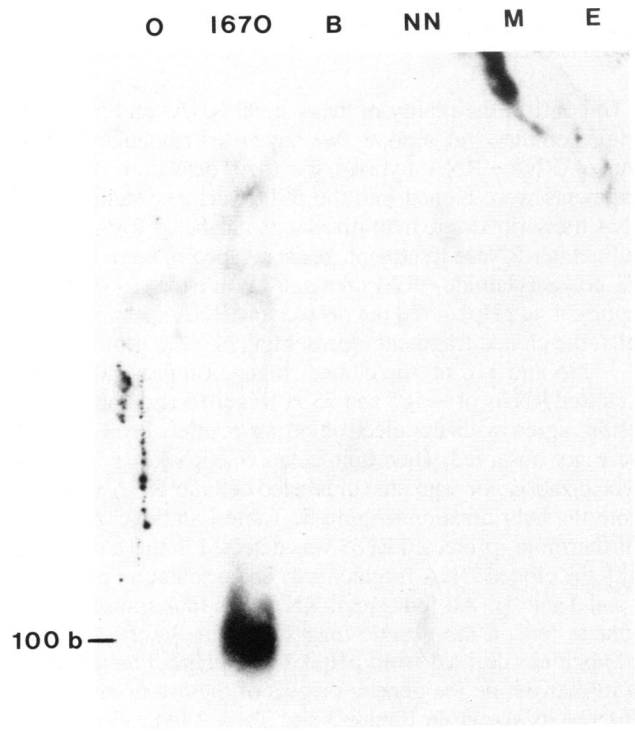

Fig. 3. Northern blot of RNAs from different cell lines and tissues. RNA (10 μg) was electrophoresed on a 1.1% agarose gel and blotted to nitrocellulose. The blot was then incubated with radiolabelled pHp2.5 probe. O, owl monkey kidney cell line; 1670, herpes virus saimiri tumor cell line; B, brain tissue from lymphoma bearing animal; NN, lymph node tissue from healthy uninfected owl monkey; M, molt-3 cell line; E, EBV transformed owl monkey cell line (VII).

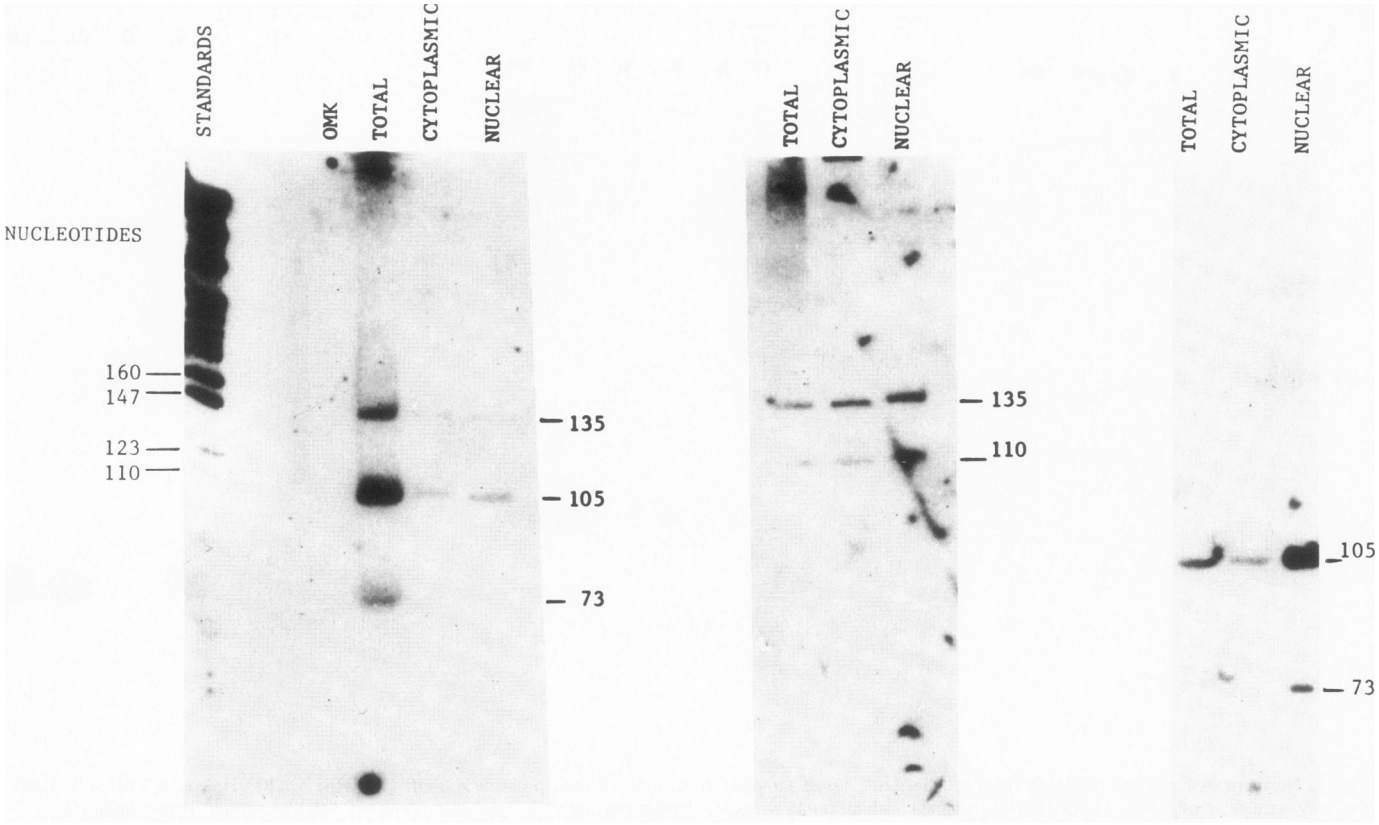

Fig. 4. Separation of herpes virus saimiri tumor cell RNAs by polyacrylamide gel electrophoresis. Total or fractionated 1670 cell RNA was electrophoresed through an 8% polyacrylamide−8 M urea gel, electroblotted to nylon membrane and hybridized with ^{32}P-labeled (i) pT7.4; (ii) pHp3.1; (iii) pHp2.5. Standards are the *Hae*III fragments of pBR322 end-labeled with ^{32}P by the Klenow fragment of DNA polymerase; standards were denatured with formamide prior to electrophoresis.

To confirm the reality of these small RNAs and to map their origins on the viral genome, we have used ribonuclease protection of RNA−RNA hybrids for their detection. Viral DNA fragments were cloned into the pSP6 vector system and [^{32}P]-RNA transcripts were hybridized with unlabeled RNA from 1670 cells. After RNase treatment, resistant species were resolved on 8% polyacrylamide−8 M urea gels. While the cloned fragment representing pHp1.4 did not protect any RNA species from 1670 cells, the cloned fragment representing pHp3.1 protected RNAs of ~135 and 110 nt; the cloned fragment representing pHp2.5 protected RNAs of ~105 and 73 nt (Figure 5 and Table I). These results agree with the electroblotting results. Protected RNAs were not observed when uninfected cell RNA was used in the hybridization nor when the unlabeled cellular RNA was omitted from the hybridization (Figure 5, Table I and data not shown). Furthermore, protected RNA was detected in these experiments with the cloned DNA fragments in one orientation only (Figure 5 and Table I). All four small RNAs are thus transcribed from right to left on the genetic map shown in Figure 1.

Subclones derived from pHp3.1 and pHp2.5 have been used to further define the genetic origins of these four small RNAs. The results shown in Figure 5 and Table I indicate that: (i) the 135-nt species is derived from sequences between the *Hpa*II site at 1.5 kbp and the *Hin*dIII site at 2.1 kbp; (ii) the 110-nt species is derived from sequences between the *Hin*dIII site at 2.1 kbp and the *Bgl*II site at 3.1 kbp; (iii) the 105- and 73-nt species are derived from sequences between the *Hpa*II site at 4.8 kbp and the *Hin*dIII site at 5.9 kbp.

Table I. Summary of ribonuclease protection experiments[a]

Lane	Total cell RNA used in hybridization	Map units for making ^{32}P-transcript probe[b]	Orientation of ^{32}P probe[c]	RNA detected[d]
2	OMK	0.1−1.4 (M−M)	Left−right	None
3	OMK	0.1−1.4 (M−M)	Right−left	None
4	1670	0.1−1.4 (M−M)	Left−right	None
5	1670	0.1−1.4 (M−M)	Right−left	None
6	OMK	1.4−4.2 (M−M)	Left−right	None
7	OMK	1.4−4.2 (M−M)	Right−left	None
8	1670	1.4−4.2 (M−M)	Left−right	135, 110
9	1670	1.4−4.2 (M−M)	Right−left	None
10	OMK	1.4−2.9 (M−B)	Left−right	None
11	1670	1.4−2.9 (M−B)	Left−right	135, 110
12	OMK	1.4−1.9 (M−D)	Left−right	None
13	1670	1.4−1.9 (M−D)	Left−right	135
14	OMK	4.2−6.5 (M−M)	Left−right	None
15	OMK	4.2−6.5 (M−M)	Right−left	None
16	1670	4.2−6.5 (M−M)	Left−right	105, 73
17	1670	4.2−6.5 (M−M)	Right−right	None
18	OMK	4.2−5.4 (M−D)	Left−right	None
19	1670	4.2−5.4 (M−D)	Left−right	105, 73

[a]Table I shows the origin of materials used in Figure 5.
[b]Numbers refer to the viral DNA map units of pSp6 cloned fragments and the letters refer to the restriction endonuclease sites at these map units. M, *Hpa*II; B, *Bgl*II; D, *Hin*dIII.
[c]This refers to the 5′−3′ orientation (Figure 1) of the ^{32}P-labeled RNA used in hybridization.
[d]Size of RNA in nucleotides.

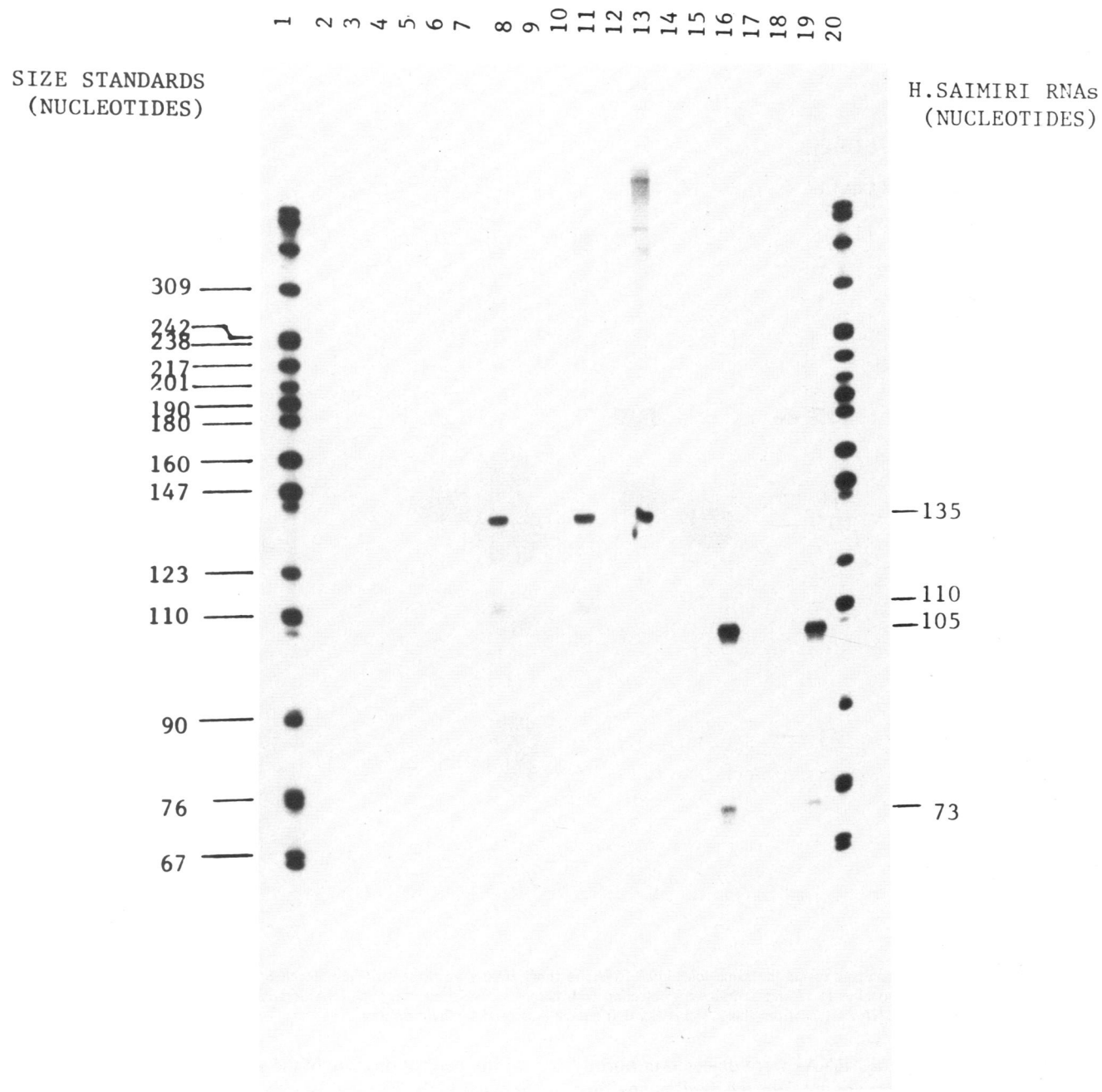

Fig. 5. Detection of ribonuclease resistant hybrids following hybridization of 1670 tumor cell RNA with ^{32}P-labeled RNA probes. The RNAs used in hybridization are indicated according to the lanes in Table 1.

RNA prepared from owl monkey tissues of three different strain 11-induced lymphomas was analyzed in a similar manner. RNA from these samples was hybridized with ^{32}P-transcripts made in the SP6 system and RNase resistant hybrids were resolved by polyacrylamide gel electrophoresis. Four RNAs of the same size detected in 1670 cells were also detected in these lymphoma samples (Figure 6). Thus, synthesis of these four small RNAs appears to be a general property of lymphoid cells from strain 11 herpes virus saimiri-induced tumors.

To obtain preliminary information on cellular localization, cells were fractionated into nuclei and cytoplasm following lysis with detergent. Aliquots of nuclear and cytoplasmic RNA derived from equivalent numbers of cells were analyzed by electroblot-hybridization and by ribonuclease protection (Figures 4 and 7). In four determinations, 30–90% of the 135-, 105- and 73-nt species were found in the nuclear fraction.

Discussion

In summary, we have documented the presence of four small RNAs of ~73, 105, 110 and 135 nt in lymphoid tumor cells transformed by herpes virus saimiri strain 11. These four small RNAs were detected in each of four different animals with virus-induced lymphoid tumors and in the 1670 tumor cell line. The genetic origins of these small RNAs were mapped to 0.5–1.2 kbp restriction fragments within the left-most 7.4 kbp of L-DNA. The synthesis of these small RNAs as well as the larger poly(A)-containing RNAs derived from this region is under

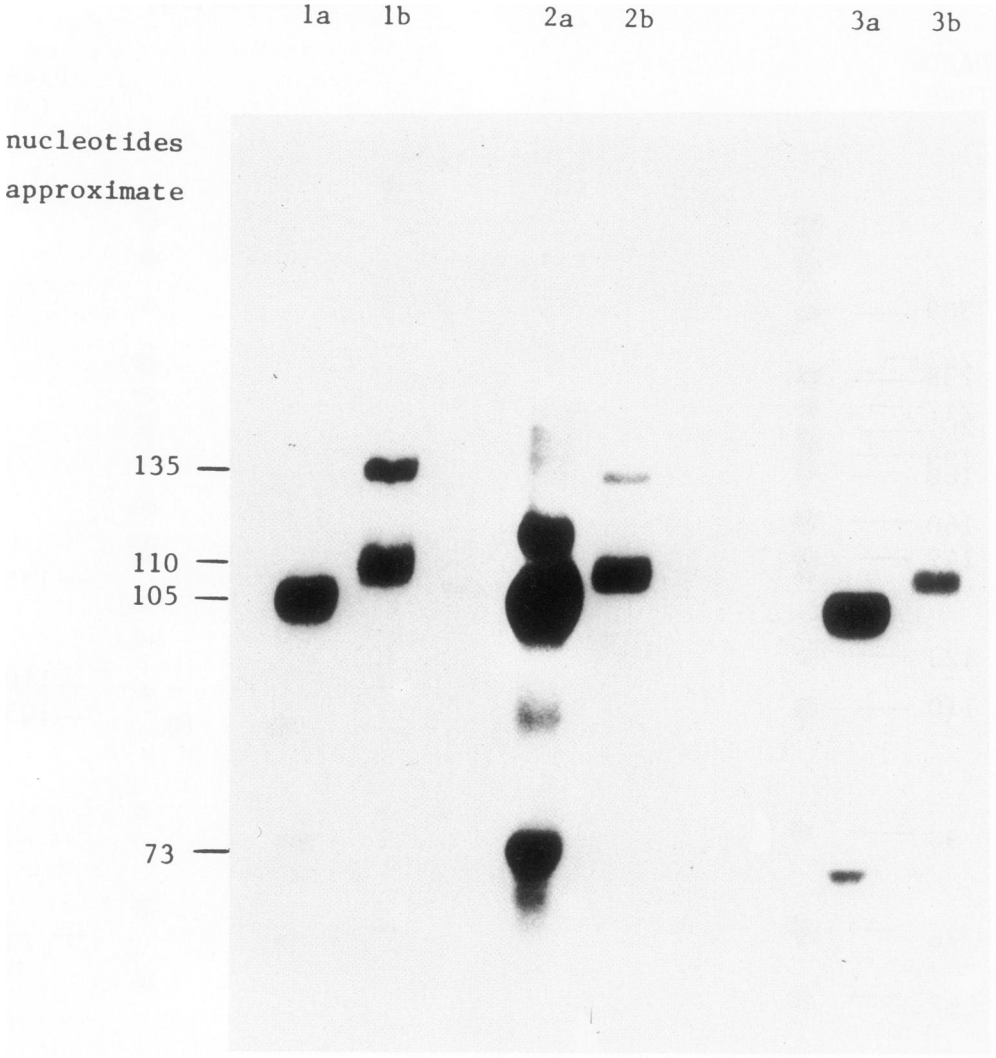

Fig. 6. Ribonuclease protection analyses reveal the same four small RNAs in fresh tumors as in the 1670 tumor cell line. RNA was prepared from tumor material of three different owl monekys (1, 2 and 3) following infection with herpes virus saimiri. (i) [^{32}P]RNA derived from 3.1 kbp *Hpa*II fragment was used for hybridization; (ii) [^{32}P]RNA derived from the 2.5 kbp *Hpa*II fragment was used for hybridization.

stringent regulation. The small RNAs were detected uniformly in lymphoid tumor cells but were not detected at all at any time point during the course of lytic infection of OMK cells. Conversely, the poly(A)-containing RNAs from this region detected in infected OMK cells were not detected at all in the four lymphoid tumors nor in the 1670 tumor cell line. While the molecular mechanisms underlying this stringent regulation have not been identified, we might expect viral *cis*-acting enhancer signals and *trans*-acting regulatory molecules to function in a cell-type specific manner.

In a separate study, a single herpes virus saimiri-specified polyadenylated RNA was detectd in the 1670 tumor cell line (Knust *et al.*, 1983); this polyadenylated RNA was derived from sequences near the right end of L-DNA. Subsequent analyses revealed this sequence to code for functional thymidylate synthetase (Honess *et al.*, 1986). Other poly(A)-containing transcripts were not detected in 1670 cells in the study of Knust *et al.* (1983). Thus extensive expression of the herpes virus saimiri genome is apparently not necessary to maintain the transformed phenotype.

Fifty percent or more of the 73-, 105- and 135-nt species fractionated with the nucleus in most determinations. Since NP40 detergent lysis was used for the fractionations, these three RNAs probably are primarily nuclear in location. However, *in situ* hybridization procedures may be better suited for addressing this issue.

Three herpes virus saimiri mutants with deletions in this region have been tested previously for transforming activity and oncogenicity (Desrosiers *et al.*, 1985; Desrosiers *et al.*, 1986). The results of these studies correlate interestingly with the genetic origins of the small RNAs (Figure 1). The S4 mutant deletes sequences encoding the 135 and 110 nt RNAs, while the 11att mutant potentially deletes sequences for the 135-nt RNA only; both the 11att and the S4 deletion mutants score negative for *in vitro* transformation and oncogenicity. However, the KH deletion falls outside the coding sequences for the small RNAs, and the KH mutant of herpes virus saimiri retains the ability to transform T cells and induce lymphoma. We are currently using several approaches to try to demonstrate directly that synthesis of one or more of the small RNAs is essential for transforma-

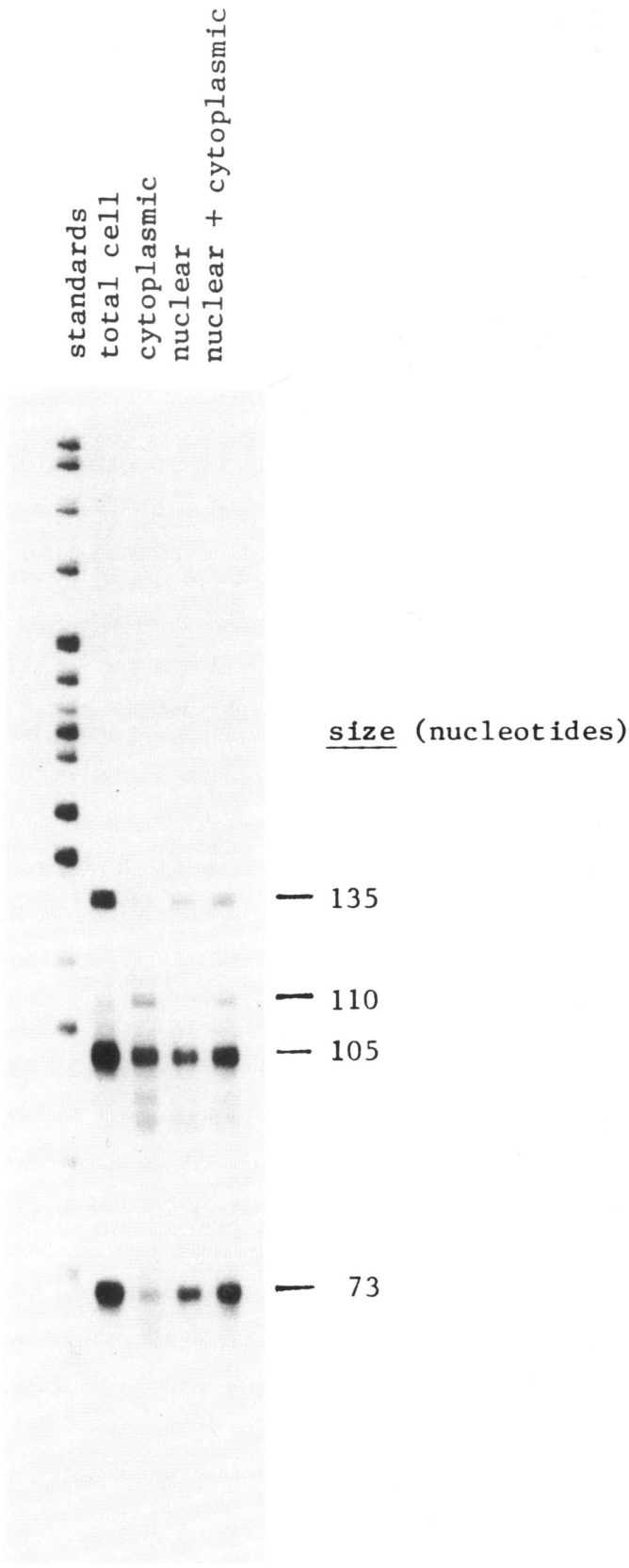

Fig. 7. Detection of RNA in subcellular fractions by ribonuclease protection. RNA from sub-cellular fractions derived from an equivalent number of 1670 cells was used for the ribonuclease protection assay. 'Nuclear + cytoplasmic' represents a mix of these RNAs from equivalent numbers of cells. Total is total cell RNA. Equal c.p.m. of [^{32}P]RNA derived from both the 3.1 and 2.5 kbp *Hpa*II fragments were mixed for the hybridization.

tion. If small RNA is directly involved in transformation, it would be a novel feature for viral oncogenesis.

What is known about other viral and cellular small RNAs and what might the function of the small herpes virus saimiri RNAs be? Epstein–Barr virus (EBV) codes for two small RNAs called EBER1 and EBER2 (Lerner *et al.*, 1981; Arrand and Rymo, 1982); however, it is not known if they are dispensable for replication, if they are essential for immortalization and whether they are functionally similar to any of the herpes virus saimiri small RNAs. Adenovirus codes for two small RNAs called VAI and VAII (Reich *et al.*, 1966; Soderlund *et al.*, 1976); these RNAs are not essential for transformation. VAI apparently prevents phosphorylation of eIF-2 after infection (Schneider *et al.*, 1985; O'Malley *et al.*, 1986) and functional substitution of VAI by the EBER RNAs has been reported (Bhat and Thimmappaya, 1985). Additionally, several examples can be cited where small RNAs have been shown or suggested to be important determinants regulating expression: (i) the small leader RNA of vesicular stomatitis virus is responsible for inhibiting cellular transcription (Grinnell and Wagner, 1985); (ii) small RNAs have been found to play an essential role in the processing of RNA transcripts in the nucleus, and regulation through differential processing has been proposed (Turner, 1985); (iii) small RNAs have been identified as tissue-specific transcripts [coding sequences for these small RNAs have been described as components of controlling elements called 'identifier' sequences (Sutcliffe *et al.*, 1982, 1984a, b; Milner *et al.*, 1984)]. Coding sequences for these small RNAs occur within the introns of genes whose expression is tissue-specific (Barta *et al.*, 1981; Milner *et al.*, 1984; Gutierrez-Hartmann *et al.*, 1984). DNA sequencing and further mapping will be needed to determine whether the 135 and 110 nt RNAs are derived from the intron sequences (Kamine *et al.*, 1984; Figure 1).

If the small herpes virus saimiri RNAs are analogous to one of the classes of small RNAs discussed above, the herpes virus saimiri system may be ideal for unravelling how such RNAs work. One possibility that might be considered is whether the small herpes virus saimiri RNAs activate expression of certain products important for the regulation of T-cell growth. It should be noted that the activation of transcription of specific cellular genes seems to occur during transformation by at least some DNA viruses, for example SV40 (Schutzbank *et al.*, 1982; Singh *et al.*, 1985) and EBV (Cheah *et al.*, 1986).

Materials and methods

Cells

OMK cells (line 637) were grown in 150 cm^2 flasks containing MEM supplemented with 10% heat-inactivated fetal calf serum, penicillin and streptomycin. Upon reaching confluence herpes virus saimiri strain 11 virus was added at a multiplicity of infection of one. Molt-3 cells, 1670 cells, and an EBV-transformed owl monkey B-cell line (VII) were grown in stationary suspension cultures in RPMI medium supplemented with 10% heat-inactivated fetal calf serum. Tumor biopsy samples were obtained from four owl monkeys (*Aotus trivirgatus*) that had been inoculated intramuscularly with 10^5 infectious particles of herpes virus saimiri strain 11. Three to five weeks after inoculation, the monkeys became moribund and, at death, tissue samples of peripheral lymph nodes, brain, liver, spleen and peripheral blood lymphocytes were taken for the immediate preparation of cellular RNA and for histological analysis. Histopathologic examination was performed by Dr N.W.King, New England Regional Primate Research Center (Desrosiers *et al.*, 1985).

RNA isolation

RNA was isolated from herpes virus saimiri strain 11 infected OMK cells by guanidine thiocyanate extraction and precipitation as previously described (Kamine *et al.*, 1984). Molt-3 cells, 1670 cells and EBV-transformed lymphoid cells (VII) were pelleted by centrifugation and RNA was extracted by the same guanidine thiocyanate procedure. In each case ~1 g of cells was extracted per 30 ml of

extraction buffer. RNA from fresh tissue samples was prepared in the same manner by directly homogenizing the tissue in 30 ml of extraction buffer in a Omnimixer (Kamine *et al.*, 1984). Nuclear and cytoplasmic fractions for RNA isolation were prepared following NP-40 lysis of cells in hypotonic buffer as described in Maniatis *et al.* (1982); the nuclear pellet was washed once with the NP-40 buffer prior to extraction of RNA. RNA was isolated from cytoplasmic and DNase-treated nuclear fractions by phenol-chloroform extractions.

Northern blot hybridization

For the data shown in Figures 2 and 3, total cellular RNA was denatured with glyoxal (McMaster and Carmichael, 1977). The indicated amounts of RNA were then electrophoresed through agarose gels and transferred to nitrocellulose as described by Thomas (1980). Blots were prehybridized and the bound RNA was hybridized to radiolabeled DNA in the presence of a 50% formamide and 10% dextran sulfate buffer as previously described (Kamine *et al.*, 1984). Radiolabeled DNA was prepared by nick translation with [α-^{32}P]dATP and [α-^{32}P]TTP essentially as described by Rigby *et al.* (1977). The sp. act. of the DNAs were $2 \times 10^8 - 4 \times 10^8$ d.p.m./μg of DNA. The blots were stripped of ^{32}P-labeled probe for sequential hybridization by incubating them in 50% formamide for 1 h at 60°C, after which the cycle of prehybridization and rehybridization to radiolabeled DNA was repeated.

For electroblotting (Figure 4), total cytoplasmic and nuclear RNAs were electrophoresed through 8% polyacrylamide gels containing 8 M urea (15 × 15 cm). RNA was electroblotted (Hoeffer Scientific, San Francisco, California) onto 0.1 μm Nytran membrane filters (Schleicher and Schuell, Keene, NH) at 0.5 A for 1.5–2 h in TAE buffer (10 mM Tris-base, 5 mM sodium acetate, 0.5 mM EDTA). After electroblotting, filters were baked at 80°C for 2 h and pre-hybridized in 50% formamide, 5 × Denhardt's reagent, 0.1% SDS, 5 × SSPE and 500 μg/ml carrier DNA at 42°C. Hybridization with ^{32}P-labeled DNA probes was performed at 42°C in 50% formamide, 2 × Denhardt's solution, 0.1% SDS, 200 μg/ml carrier DNA and 5 × SSC for 12–16 h. The filters were exposed to film at −70°C.

Molecular cloning

*Hpa*II fragments of 2.5 and 3.1 kbp derived from pT7.4 (see Figure 1) were cloned into the *Acc*I site of the plasmids pSp64 and pSp65 (Promega Biotec, Madison, Wis.). Plasmids were obtained with herpes virus saimiri DNA inserts in both orientations with respect to the Sp6 promoter. The *Hpa*II fragment of 1.4 kbp from pT7.4 was cloned similarly into pGEM-2 (Promega Biotec), a cloning vector containing promoters for Sp6 and T7 RNA polymerases at either side of the polylinker. Similarly, subclones derived from the 3.1- and 2.5-kbp fragments (Table I) were obtained using one of these vectors containing Sp6 and/or T7 polymerase promoters.

Ribonuclease protection

^{32}P-labeled RNA was synthesized using Sp6 or T7 promoters depending on the clone and its orientation; the reaction conditions have been described elsewhere (Melton *et al.*, 1984). In a typical reaction 50 μCi of [^{32}P]UTP or [^{32}P]CTP (Sp6 grade, Amersham, Arlington Heights, IL) was used, maintaining the other NTP concentrations at 500 μM. After 20 min incubation at 42°C, the concentration of UTP or CTP was brought to 500 μM by the addition of unlabeled UTP or CTP and the reaction was allowed to proceed for another 40 min. Removal of the DNA template and processing of the RNA was performed as described previously (Melton *et al.*, 1984). The tumor RNA and ^{32}P-labeled RNA were dissolved directly in the hybridization buffer (80% formamide, 40 mM Pipes, 400 mM NaCl and 1 mM EDTA). In a typical hybridization reaction, 20 μg of 1670 tumor cell RNA was hybridized with 5×10^5 d.p.m. of [^{32}P]RNA made by the bacteriophage RNA polymerase in a final volume of 30 μl. The samples were heated at 85°C for 10 min and transferred immediately to a water bath at 45°C for overnight incubation. After hybridization, 350 μl of 300 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, pH 7.5, containing 40 μg/ml pancreatic ribonuclease A and 2 μg/ml ribonuclease T1 was added; this was incubated at room temperature for 30 min. Proteinase K (50 μg) and 10 μl of 20% SDS were added and incubated for 15 min at 37°C. The RNA was then extracted with phenol and chloroform and precipitated with ethanol. Samples were dried under vacuum, resuspended in 2 μl running dye with 80% formamide, heated to 85°C for 10 min and applied onto a 8% polyacrylamide−8 M urea sequencing gel. Following electrophoresis, gels were covered with Saran Wrap and exposed to film.

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References

Arrand,J.R. and Rymo,L. (1982) *J. Virol.*, **41**, 376–389.
Barta,A., Richards,R.I., Baxter,J.D. and Shine,J. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4867–4871.
Bhat,R.A. and Thimmappaya,B. (1985) *J. Virol.*, **56**, 750–756.
Cheah,M.S.C., Ley,T.J., Tronick,S.R. and Robbins,K.C. (1986) *Nature*, **319**, 238–240.
Desrosiers,R.C., Burghoff,R.L., Bakker,A. and Kamine,J. (1984) *J. Virol.*, **49**, 343–348.
Desrosiers,R.C., Bakker,A., Kamine,J., Falk,L.A., Hunt,R.D. and King,N.W. (1985) *Science*, **228**, 184–187.
Desrosiers,R.C., Silva,D.P., Waldron,L.M. and Letvin,N.L. (1986) *J. Virol.*, **57**, 701–705.
Falk,L., Silva,D., Byington,R. and Schooley,R. (1983) In Parker,J.W. and O'Brien,R.L. (eds), *Intercellular Communication in Leucocyte Function*. John Wiley & Sons Inc., NY, pp. 577–580.
Fleckenstein,B. and Desrosiers,R.C. (1982) In Roizman,B. (ed.), *The Herpesviruses*. Plenum Publishing Corporation, NY, pp. 253–332.
Grinnell,B.W. and Wagner,R.R. (1985) *Mol. Cell. Biol.*, **5**, 2502–2513.
Gutierrez-Hartmann,A., Lieberburg,I., Gardner,D., Baxter,J.D. and Cathala,G.G. (1984) *Nucleic Acids Res.*, **12**, 7153–7173.
Honess,R.W., Brodemer,W., Cameron,K.R., Fleckenstein,B. and Randall,R.E. (1986) *Proc. Natl. Acad. Sci. USA*, in press.
Kamine,J., Bakker,A. and Desrosiers,R.C. (1984) *J. Virol.*, **52**, 532–540.
Knust,E., Dietrich,W., Fleckenstein,B. and Bodemer,W. (1983) *J. Virol.*, **48**, 377–383.
Koomey,J.M., Mulder,C., Burghoff,R.L., Fleckenstein,B. and Desrosiers,R.C. (1984) *J. Virol.*, **50**, 662–665.
Lerner,M.R., Andrews,N.C., Miller,G. and Steitz,J.A. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 805–809.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
McMaster,G.K. and Carmichael,G.G. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 4835–4838.
Melton,D.A., Krieg,P.A., Rebagliati,M.R., Maniatis,T., Zinn,K. and Green, M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.
Milner,R.J., Bloom,F.E., Lai,C., Lerner,R.A. and Sutcliffe,J.G. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 713–717.
O'Malley,R.P., Mariano,T.M., Siekierke,J. and Mathews,M.B. (1986) *Cell*, **44**, 391–400.
Rabin,H., Hopkins,R.F., Desrosiers,R.C., Ortaldo,J.R., Djeu,J.Y. and Neubauer,R.H. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 4563–4567.
Reich,P.R., Forget,B. and Weissman,S.M. (1966) *J. Mol. Biol.*, **17**, 428–439.
Rigby,P.W.J., Dieckmann,M., Rhodes,C. and Berg,P. (1977) *J. Mol. Biol.*, **113**, 237–251.
Schaffer,P.A., Falk,L.A. and Deinhardt,F. (1975) *J. Natl. Cancer Inst.*, **55**, 1243–1246.
Schirm,S., Muller,I., Desrosiers,R.C. and Fleckenstein,B (1984) *J. Virol.*, **49**, 938–946.
Schneider,R.J., Safer,B., Munemitsu,S.M., Samuel,C.E. and Shenk,T. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4321–4325.
Schutzbank,T., Robinson,R., Oren,M. and Levine,A.J. (1982) *Cell*, **30**, 481–490.
Singh,K., Carey,M., Saragosti,S. and Botchan,M. (1985) *Nature*, **314**, 553–556.
Soderlund,H., Pettersson,U., Vennstrom,B., Philipson,L. and Mathews,M.B. (1976) *Cell*, **7**, 585–593.
Sutcliffe,J.G., Milner,R.J., Bloom,F.E. and Lerner,R.A. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4942–4946.
Sutcliffe,J.G., Milner,R.J., Gottesfeld,J.M. and Lerner,R.A. (1984a) *Nature*, **308**, 237–241.
Sutcliffe,J.G., Milner,R.J., Gottesfeld,J.M. and Reynolds,W. (1984b) *Science*, **225**, 1308–1315.
Thomas,P.S. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5201–5205.
Turner,P. (1985) *Nature*, **316**, 105–106.
Wright,J.J. (1978) Ph.D. Thesis, University of Illinois Medical Center.

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