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The Human T-Lymphotropic Virus Type I *tax* Gene Can Cooperate with the *ras* Oncogene To Induce Neoplastic Transformation of Cells

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Epidemiologic studies have linked infection by the human T-lymphotropic virus type I (HTLV-I) with the development of adult T-cell leukemia. The low penetrance of the virus and the long latency for disease manifestation are factors that obscure the role of HTLV-I infection in oncogenesis. We have used an in vitro transformation assay system to determine directly whether the HTLV-I *tax* gene has transformation potential. Transfection of the *tax* gene alone into early-passage rat embryo fibroblasts did not induce morphological alterations. However, cotransfection of *tax* with the selectable marker plasmid pRSVneo gave rise to G418-resistant colonies that could be established as immortalized cell lines. Cotransfection of *tax* with the *ras* oncogene into rat embryo fibroblasts gave rise to foci of transformed cells that were highly tumorigenic in nude mice. These data represent a direct demonstration of the oncogenic potential of the *tax* gene in nonlymphoid cells and establish HTLV-I as a transforming virus.

DNA transfection studies using viral and cellular oncogenes have demonstrated a requirement of two cooperating oncogenes to transform primary rodent cells in culture (2, 13, 24, 26). Many of the known oncogenes can be categorized into one of two groups, depending on their ability to cooperate in this transformation assay (28). Members of one class of genes share the properties that their gene products localize to the cytoplasm and efficiently transform established cell lines such as NIH 3T3. Gene products of the other class localize to the nucleus, function as transcriptional regulators, and are able to immortalize primary cells. Although human T-lymphotropic virus type I (HTLV-I) infection has been shown to be associated with the development of adult T-cell leukemia (8, 21), the long latency of disease onset has prompted the suggestion that HTLV-I infection represents a necessary but insufficient step in oncogenesis. The role of the virus and specific viral genes in tumor development is not known. In this study, we used the transformation of early-passage rat embryo cells as an in vitro assay to evaluate the transforming properties of the HTLV-I *tax* gene.

Passage 1 primary rat embryo cells were obtained from Microbiological Associates. DNA transfections into passage 2 rat embryo cells were performed as previously described (23). Briefly, primary rat embryo cells were plated at 1.5×10^6 cells per T75 flask 24 h before transfection. A 15-μg amount of each plasmid was coprecipitated in 2 ml of calcium phosphate and applied to the cells for 4 h. The *ras*-containing plasmid pEJ (19), the *tax*-containing plasmid pHTLV-*tat*1 (18), and the E1a-containing plasmid pE1a (previously named pGC212 [10]) have all been previously described. In addition to the 40-kilodalton Tax protein, the mRNA derived from the pHTLV-*tat*1 plasmid also encodes the p21 protein, which is read in a different reading frame from Tax. Previous studies of HTLV-I-infected cells have shown that the p21 protein accumulates to very low levels, in contrast to the Tax protein (11, 16). This difference is probably attributable to the fact that the p21 initiation codon is located downstream of the Tax initiation codon, and the translation initiation sequence surrounding the p21 AUG does not conform closely to the Kozak consensus sequence.

In transfections in which a single gene was introduced, 15 μg of pUC18 DNA was used to maintain 30 μg of DNA per transfection. After 4 h, the cells were glycerol shocked, washed, and refed. Forty hours later, cells were trypsinized, counted, and plated in 10-cm dishes at two cell densities. Plates were refed every 5 days. After 2 to 3 weeks, either (i) individual foci were picked with cloning cylinders and expanded to form cell lines or (ii) the plates were stained with a solution of ethanol–phosphate-buffered saline–formalde-

TABLE 1. Focus formation in rat embryo cells after oncogene transfection[a]

Transfected DNA	Expt no.	No. of foci/ no. of cells (10^6) plated
None	1	0/2.5 (0)
	2	0/2.5 (0)
	3	0/1.0 (0)
tax	1	0/2.5 (0)
	2	0/3.0 (0)
ras	1	0/1.3 (0)
	2	0/2.8 (0)
	3	0/2.0 (0)
tax + E1a	1	0/3.5 (0)
tax + *ras*	1	177/1.3 (146)
	2	66/3.2 (21)
	3	28/2.0 (14)
E1a + *ras*	1	326/2.3 (145)
	2	200/3.4 (59)
	3	46/1.0 (46)

[a] Oncogenes were introduced into rat embryo cells by calcium phosphate-DNA coprecipitation. Approximately 2 weeks after transfection, the plates were stained with crystal violet and the number of foci was determined. Each number in parentheses represents the number of foci per 10^6 cells plated.

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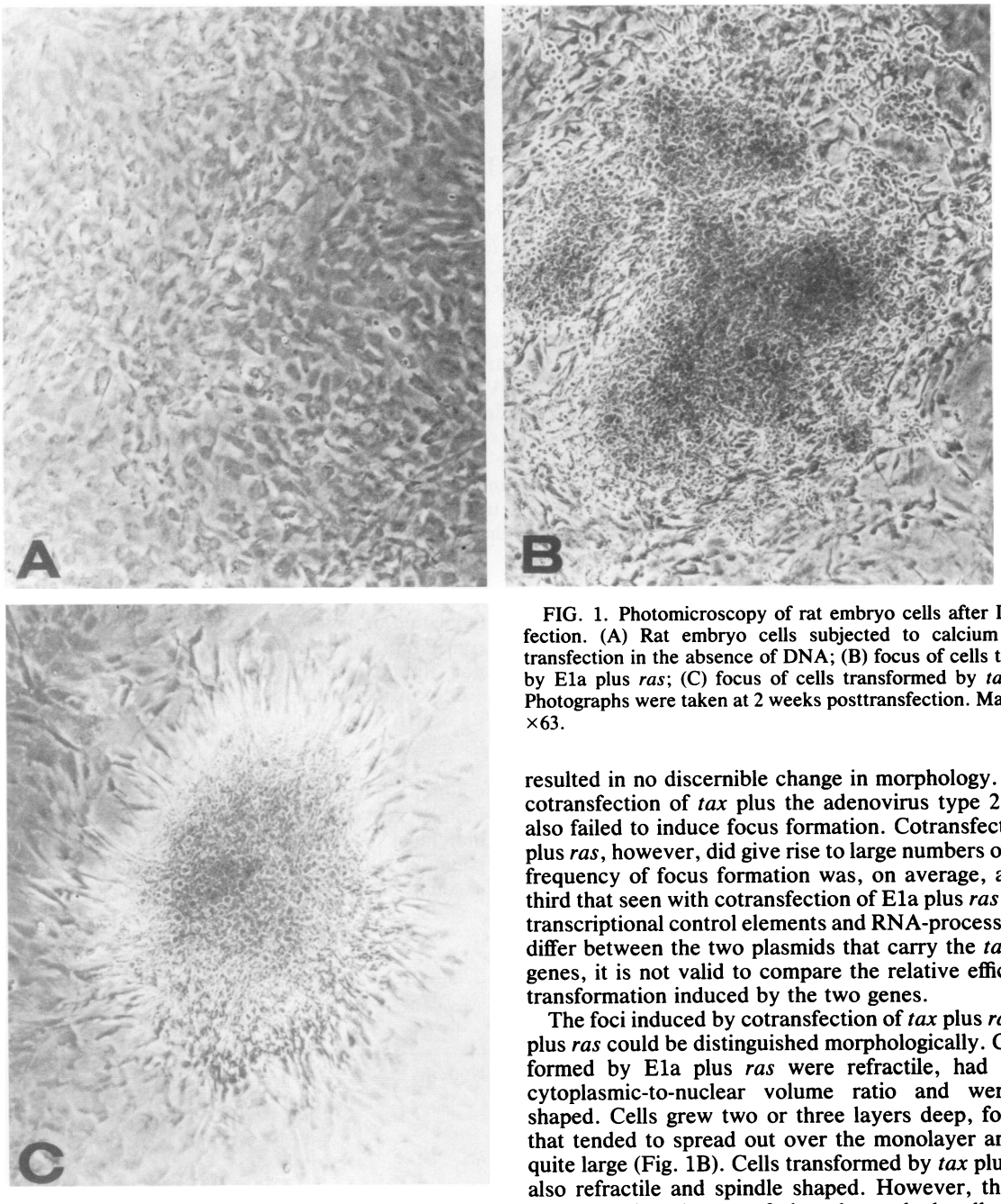

FIG. 1. Photomicroscopy of rat embryo cells after DNA transfection. (A) Rat embryo cells subjected to calcium phosphate transfection in the absence of DNA; (B) focus of cells transformed by E1a plus *ras*; (C) focus of cells transformed by *tax* plus *ras*. Photographs were taken at 2 weeks posttransfection. Magnification, ×63.

hyde (50:45:5) containing 0.25% crystal violet dye, and the number of foci was determined.

Previously, we and others have described obtaining transformed cell lines from primary rodent cells by cotransfecting the *ras* oncogene with a selectable marker gene such as *neo* (23, 27). Transformation of primary rodent cells by *ras* alone occurs only when the untransfected cells are killed by selection (12). In the absence of selection, focus formation on a monolayer of primary cells requires the action of two cooperating oncogenes (12, 13, 26).

The effects of transfecting *tax* alone and in combination with other oncogenes into early-passage rat embryo fibroblasts are shown in Table 1. Transfection of *tax* or *ras* alone resulted in no discernible change in morphology. Similarly, cotransfection of *tax* plus the adenovirus type 2 E1a gene also failed to induce focus formation. Cotransfection of *tax* plus *ras*, however, did give rise to large numbers of foci. The frequency of focus formation was, on average, about one-third that seen with cotransfection of E1a plus *ras*. Since the transcriptional control elements and RNA-processing signals differ between the two plasmids that carry the *tax* and E1a genes, it is not valid to compare the relative efficiencies of transformation induced by the two genes.

The foci induced by cotransfection of *tax* plus *ras* and E1a plus *ras* could be distinguished morphologically. Cells transformed by E1a plus *ras* were refractile, had a reduced cytoplasmic-to-nuclear volume ratio and were spindle shaped. Cells grew two or three layers deep, forming foci that tended to spread out over the monolayer and become quite large (Fig. 1B). Cells transformed by *tax* plus *ras* were also refractile and spindle shaped. However, the foci featured multiple layers of densely packed cells and were smaller than foci induced by E1a plus *ras* (Fig. 1C).

Five cell lines from independent foci transformed by *tax* plus *ras* were established and examined for expression of the transfected genes. Northern (RNA) blot analysis of cytoplasmic RNA revealed that all five lines expressed abundant levels of the *ras* oncogene (Fig. 2A). No significant difference in the level of *ras* RNA was seen when the *tax*-plus-*ras*-transformed cell lines were compared with the E1a-plus-*ras*-transformed lines (data not shown). This result suggests that the ability of *tax* to cooperate with *ras* is not due simply to a stimulation of the level of expression of *ras*. Results of Western blot (immunoblot) analysis (Fig. 2B) showed that all five transformed lines expressed the 40-kilodalton Tax protein.

To test the tumorigenic potential of these cell lines, 5 ×

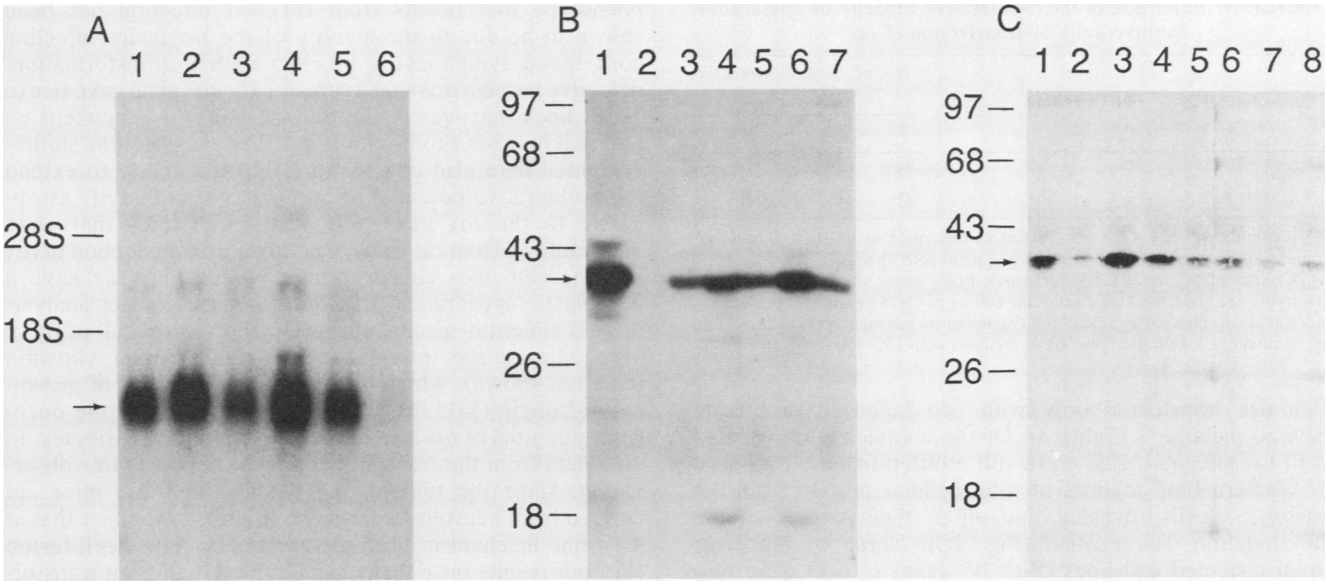

FIG. 2. Expression of transfected genes in rat embryo cell lines. (A) Northern blot analysis of *ras* expression in cell lines transformed by *tax* plus *ras*. Cytoplasmic RNA (15 μg) was electrophoresed in a denaturing agarose gel, blotted to nitrocellulose, and hybridized with a *ras*-specific probe. Lanes: 1 to 5, five independently isolated rat embryo cell lines transformed by *tax* plus *ras*; 6, cytoplasmic RNA from normal passage 3 rat embryo cells. (B) Western blot analysis of Tax expression in transformed cell lines. Cells in 10-cm-diameter dishes were lysed in RIPA buffer, immunoprecipitated, and subjected to Western blot analysis with Tax antiserum (4). Lanes: 1, PX-1, a cell line established from a neurofibroma that developed in a mouse harboring the *tax* gene (18); 2, EJ/E1a-4, a rat embryo cell line transformed by *ras* plus E1a; 3 to 7, five rat embryo cell lines transformed by *tax* plus *ras*, corresponding to lanes 1 to 5 of panel A. (C) Western blot analysis of Tax expression in the eight clones of passage 5 rat embryo cells cotransfected with pRSVneo plus pHTLV-*tat*1 described in Table 3. Sample preparation was as described for panel B.

10^5 cells were injected subcutaneously into the flanks of 5-week-old NCR-nu nude mice (Table 2). Five mice were injected for each cell line. All cell lines gave rise to palpable tumors in all injected mice within 7 days. Tumors continued to increase in size and proved to be lethal at approximately 4 to 5 weeks postinjection. Autopsies were performed on two mice from each of the five groups. Histological examination of tissue samples revealed that all 10 mice had metastatic tumors in the inguinal lymph nodes, indicating that these cell lines displayed a malignant phenotype in nude mice (data not shown). All five cell lines formed tumors that were fibrosarcomalike in appearance.

Another property that is frequently associated with *trans*-activator genes with transformation potential is the ability to immortalize primary cells in culture (1, 9, 25). We assayed the ability of the *tax* gene to extend the proliferative capacity of early-passage rat embryo fibroblasts by cotransfecting *tax* and the selectable marker plasmid pRSVneo (5). After selection with the antibiotic G418, resistant colonies were picked and expanded in an attempt to establish cell lines.

TABLE 2. Tumorigenicity of cell lines transfected with *tax* plus *ras* or *tax* plus *neo*[a]

Cell line	Value after injection of:					
	5×10^5 cells			5×10^6 cells		
	Incidence[b]	Time (wk) of:		Incidence	Time (wk) of:	
		Tumor onset[c]	Death[d]		Tumor onset	Death
Normal rat embryo fibroblasts	0/5	NA[e]	NA	0/5	NA	NA
tax + *ras*-1	5/5	<1	5	ND[f]		
tax + *ras*-2	5/5	<1	4	ND		
tax + *ras*-3	5/5	1	5	ND		
tax + *ras*-4	5/5	<1	4	ND		
tax + *ras*-5	5/5	<1	4	ND		
tax + *neo*-1	0/5	NA	NA	0/5	NA	NA
tax + *neo*-2	1/5	8	ND	2/2	22	ND
tax + *neo*-3	0/5	NA	NA	0/5	NA	NA

[a] Monolayer cultures of cells were injected subcutaneously into the flanks of 5-week-old female athymic NCR-nu nude mice obtained from the NCI Frederick Cancer Research Facility as previously described (23).
[b] Number of mice that developed tumors/number injected.
[c] Time postinjection when tumors became macroscopically visible.
[d] Time postinjection when the first mouse died as a result of tumor burden. The remaining four mice were euthanized that day.
[e] NA, Not applicable.
[f] ND, Not determined.

TABLE 3. Extension of the proliferative capacity of primary rat embryo cells by transfection of *tax*[a]

Transfected DNA	No. of colonies picked	No. of viable clones after passage no.:				
		1	2	3	4	5
neo	32	28	18	4	0	0
neo + *tax*	36	28	21	8	8	8

[a] Rat embryo cells were transfected as described in the text. The DNA content of the transfection mixtures was 3 μg of pRSVneo (*neo*) mixed with 30 μg of either pUC18 or pHTLV-*tat*1 (*tax*). Forty hours after transfection, the cells were split into medium containing 800 μg of G418 per ml. After 2 weeks, individual colonies were picked and expanded by passage every 7 days at an approximately 1:4 dilution into fresh medium with 10% fetal calf serum.

Colonies transfected only with *neo* failed to proliferate beyond passage 3 (Table 3). Of the colonies cotransfected with *tax* plus *neo*, 22% were still proliferating after passage 5. Western blot analysis of total cellular protein from the passage 5 cells revealed that all of them expressed the 40-kilodalton Tax protein (Fig. 2C). Three of the eight *tax*-transfected cell lines (Fig. 2C, lanes 6 to 8) were randomly selected before Western blot analysis and passaged an additional 15 times to confirm that these cultures represented established cell lines. These data indicate that the *tax* gene is able to rescue early-passage rat embryo cells from senescence.

Although the three rat embryo lines immortalized by *tax* did not appear to have a transformed morphology, their tumorigenicity was assayed by subcutaneous injection into nude mice. Cells were injected at two doses, 5×10^5 and 5×10^6, into the flanks of 4- to 5-week old female nude mice, and the animals were monitored for tumor development. Two of the three cell lines failed to give rise to tumors during the 25 weeks that the mice were observed after injection (Table 2). Five mice were injected for each cell line and each dose. The remaining cell line gave rise to a palpable tumor in one of five mice injected with 5×10^5 cells at 8 weeks postinjection. The tumor grew to a size of approximately 7 mm in diameter in 3 weeks and then failed to increase in size significantly over the next 5 weeks. When 5×10^6 cells were injected, tumors developed at 15 weeks postinjection in two of two mice. These tumors also grew remarkably slowly, reaching a size of only 5 mm in diameter by 22 weeks postinjection. Thus, the tumorigenicity of the *tax*-immortalized cell lines was radically different from that of the *ras*-plus-*tax*-transformed lines in incidence of tumor formation, latency of tumor development, rate of tumor growth, and mortality induced by tumor formation.

When the *tax* gene, under the transcriptional control of the HTLV-I long terminal repeat, was introduced into the germ line of mice, recipient animals developed neurofibromas at approximately 13 to 17 weeks of age (7, 18). Although this result demonstrates the oncogenic capacity of the *tax* gene, it is difficult to determine the role that the *tax* gene plays in the complex process of tumor development in transgenic mice. The in vitro model system provided by the transformation of primary rat embryo cells has allowed us to demonstrate that *tax* is able to cooperate with the *ras* oncogene and thereby establishes *tax* as a nuclear-type oncogene (28) with properties similar to those of other viral *trans*-activator genes (1, 2, 13, 20, 24, 26).

Infection of T cells with HTLV-I in vitro has been shown to alleviate growth factor dependence or extend the proliferative capacity of T cells derived from cord blood (15, 22) or clones of cytotoxic T lymphocytes (3). The immortalized phenotype that results from HTLV-I infection has been shown to be due to the activity of the *tax* gene. Infecting cord blood lymphocytes in vitro with a transformation-defective herpesvirus that expresses the *tax* gene gave rise to T-cell lines that were immortalized but still dependent on interleukin-2 for proliferation (6). The transfection studies presented here also demonstrate that the ability to extend the proliferative potential of primary cells is directly attributable to the *tax* gene. All of the cell lines that were immortalized from rat embryo cultures by transfection of *tax* and the cell lines cotransformed by *tax* plus *ras* have a fibroblastic appearance. In addition, Western blot analysis using a vimentin-specific antiserum showed that all of these lines express the intermediate filament protein vimentin (data not shown), which is characteristic of cells of mesenchymal origin (14). Therefore, it is apparent that the oncogenic potential of *tax* is not limited to lymphocytic tissues. In vivo data from the *tax* transgenic mice supports this observation, since the tumors that develop in these mice are derived from neurofibroblasts (7). In view of the fact that at least one mechanism of virus infection involves cell fusion (17), our results raise the possibility that malignant transformation induced by *tax* may not be restricted to lymphocytes.

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LITERATURE CITED

1. **Bedell, M., K. Jones, S. Grossman, and L. Laimins.** 1989. Identification of human papillomavirus type 18 transforming genes in immortalized and primary cells. J. Virol. **63**:1247–1255.
2. **Bernards, R., A. Houweling, P. Schier, J. Pols, and A. Van der Eb.** 1982. Characterization of cells transformed by Ad5/Ad12 hybrid early region I plasmids. Virology **120**:422–432.
3. **Faller, D., M. Crimmins, and S. Mentzer.** 1980. Human T-cell leukemia virus type I infection of $CD4^+$ or $CD8^+$ cytotoxic T-cell clones results in immortalization with retention of antigen specificity. J. Virol. **62**:2942–2950.
4. **Giam, C.-Z., M. Nerenberg, G. Khoury, and G. Jay.** 1986. Expression of the complete HTLV-I pX coding sequence as a functional protein in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **83**:7192–7196.
5. **Gorman, C., R. Padmanabhan, and B. Howard.** 1983. High efficiency DNA-mediated transformation of primate cells. Science **221**:551–553.
6. **Grassman, R., C. Dengler, I. Muller-Fleckenstein, B. Fleckenstein, K. McGuire, M.-C. Dokhelar, J. Sodroski, and W. Haseltine.** 1989. Transformation to continuous growth of primary human T lymphocytes by HTLV-I X-region genes transduced by a herpesvirus saimiri vector. Proc. Natl. Acad. Sci. USA **86**:3351–3355.
7. **Hinrichs, S., M. Nerenberg, K. Reynolds, G. Khoury, and G. Jay.** 1987. A transgenic mouse model for human neurofibromatosis. Science **237**:1340–1343.
8. **Hinuma, Y., K. Nagata, M. Hanaoka, M. Nakai, T. Matsumoto, K.-I. Kinoshita, S. Shirakawa, and I. Miyoshi.** 1981. Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. Proc. Natl. Acad. Sci. USA **78**:6476–6480.
9. **Houweling, A., P. Van den Elsen, and A. Van der Eb.** 1980. Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. Virology **105**:537–550.
10. **Imperiale, M., L. Feldman, and J. Nevin.** 1983. Activation of gene expression by adenovirus and herpesvirus regulatory genes acting in trans and by a cis-acting adenovirus enhancer element. Cell **35**:127–136.
11. **Kiyokawa, T., M. Seiki, S. Iwashita, K. Imagawa, F. Shimizu, and M. Yoshida.** 1985. p27 and p21, proteins encoded by the pX sequence of human T-cell leukemia virus type I. Proc. Natl.

Acad. Sci. USA 82:8359–8633.
12. **Land, H., A. Chen, J. Morgenstern, L. Parada, and R. Weinberg.** 1986. Behavior of *myc* and *ras* oncogenes in transformation of rat embryo fibroblasts. Mol. Cell. Biol. 6:1917–1925.
13. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature (London) 304:596–602.
14. **Lazarides, E.** 1980. Intermediate filaments as mechanical integrators of cellular space. Nature (London) 283:249–256.
15. **Miyoshi, I., I. Kubonishi, S. Yoshimoto, T. Akagi, Y. Ohtsuki, Y. Shiraishi, K. Nagata, and Y. Hinuma.** 1981. Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukemic T-cells. Nature (London) 294:770–771.
16. **Nagashima, K., M. Yoshida, and M. Seiki.** 1986. A single species of *pX* mRNA of human T-cell leukemia virus type I encodes *trans*-activator $p40^x$ and two other phosphoproteins. J. Virol. 60:394–399.
17. **Nagy, K., P. Clapham, R. Cheingsong-Popov, and R. Weis.** 1983. HTLV-I: induction of syncytia and inhibition by patients' sera. Int. J. Cancer 32:321–328.
18. **Nerenberg, M., S. Hinrichs, K. Reynolds, G. Khoury, and G. Jay.** 1987. The *tat* gene of HTLV-I induces mesenchymal tumors in transgenic mice. Science 237:1324–1329.
19. **Parada, L., C. Tabin, C. Shih, and R. Weinberg.** 1982. Human EJ bladder carcinoma oncogene is homologue of Harvery sarcoma virus *ras* gene. Nature (London) 297:474–478.
20. **Phelps, W. C., C. Yee, K. Munger, and P. Howley.** 1988. The HPV type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1a. Cell 53: 539–547.
21. **Poiesz, B., F. N. Ruscetti, A. F. Fazdar, P. A. Bunn, J. D. Minna, and R. C. Gallo.** 1980. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc. Natl. Acad. Sci. USA 77:7415–7419.
22. **Popovic, M., G. Lange-Wantzin, P. Sarin, D. Mann, and R. C. Gallo.** 1983. Transformation of human umbilical cord blood T-cells by human HTLV-I. Proc. Natl. Acad. Sci. USA 80: 5402–5406.
23. **Pozzatti, R., R. Muschel, J. Williams, R. Padmanabhan, B. Howard, L. Liotta, and G. Khoury.** 1986. Primary rat embryo cells transformed by one or two oncogenes show different metastatic potentials. Science 232:223–227.
24. **Rassoulzadegan, M., A. Cowie, A. Carr, N. Glaichenhaus, R. Kamen, and F. Cuzin.** 1982. The roles of individual polyoma virus early proteins in oncogenic transformation. Nature (London) 300:713–718.
25. **Rassoulzadegan, M., Z. Naghashfar, A. Cowie, A. Carr, M. Grisoni, R. Kamen, and F. Cuzin.** 1983. Expression of the large T protein of polyoma virus promotes the establishment in culture of "normal" rodent fibroblast cell lines. Proc. Natl. Acad. Sci. USA 80:4354–4358.
26. **Ruley, H. E.** 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature (London) 304:602–606.
27. **Spandidos, D., and N. Wilkie.** 1984. Malignant transformation of early passage rodent cells by a single mutated human oncogene. Nature (London) 310:469–475.
28. **Weinberg, R. A.** 1985. The action of oncogenes in the cytoplasm and nucleus. Science 238:770–776.