leading to underassessment of AIDS-related deaths include exclusion of pediatric patients from the estimates, the rigidity of the case definition used, lack of clinical information concerning a number of deaths, the unknown proportion of AIDS deaths not brought to the study mortuaries, and the cultural practice of seriously ill persons leaving Abidjan to die in their home area. Deaths due to pulmonary tuberculosis, the third-ranking cause of male adult death, were specifically not counted as AIDS cases, although with 50% of such cadavers testing HIV-positive, an important fraction of such deaths were probably attributable to HIV infection. Since our study assessed less than half of all Abidjan deaths over the study period, but used the whole population as denominator, true AIDS-specific mortality rates must be considerably higher than the minimum estimates quoted.

The disparity between rates of AIDS-specific mortality in Abidjan and in the industrialized world is extreme. Minimum AIDS-specific mortality rates in both men and women in Abidjan are higher than those in New York City (*13*). Maternal mortality is also strikingly more frequent than in the developed world. Twenty-eight percent of adult female deaths, and 34% of adult female YPLL, were due to AIDS, pregnancy-related conditions, or induced abortions, illustrating how reproductive health dominates the lives and deaths of women in this city (*14*).

Calculation of YPLL offers a useful way of estimating premature mortality (*9*) and of assessing the broader social impact of specific diseases. A number of other cities in Africa have been affected by AIDS for a longer period of time or have higher levels of HIV infection in their populations, or both. Although data concerning mortality due to AIDS are scarce, AIDS is likely to be the leading cause of adult death in a number of other African cities also, and the disease may already be affecting certain standard demographic parameters (*14*).

REFERENCES AND NOTES

1. P. Piot *et al.*, *Science* **239**, 573 (1988).
2. J. M. Mann *et al.*, *J. Am. Med. Assoc.* **255**, 3255 (1986).
3. K. Odehouri *et al.*, *AIDS* **3**, 509 (1989).
4. K. M. De Cock *et al.*, *Lancet* **ii**, 408 (1989).
5. World Health Organization, *Wkly. Epidemiol. Rec.* **61**, 72 (1986).
6. Serodiagnostic algorithm and criteria for test interpretation are described in K. M. De Cock *et al.*, *AIDS* **4**, 443 (1990).
7. R. Widy-Wirski *et al.*, *J. Am. Med. Assoc.* **260**, 3286 (1988).
8. B. Barrere, Estimation de la structure par age et sexe, population de la ville d'Abidjan—Population des quartiers d'Abidjan. Rapport complementaire, March 1989. Unpublished background document available on request.
9. Centers for Disease Control, *Morb. Mortal. Wkly. Rep.* **35**, 1S (1986).
10. For the United States, YPLL are calculated from deaths before a predetermined end point set at 65 years (*9*). Since life expectancy in the Ivory Coast is between 50 and 55 years for men and women, and official retirement is at 55 years, YPLL in this study have been calculated based on an expected age of 55 years.
11. B. Barrere, Estimation de la structure par age et sexe; Population de la ville d'Abidjan—Population des quartiers d'Abidjan, December 1988. Unpublished background document available on request.
12. ———, unpublished estimations of mortality rates in Abidjan.
13. J. W. Curran *et al.*, *Science* **239**, 610 (1988).
14. A. Rosenfield, *J. Am. Med. Assoc.* **262**, 376 (1989).
15. Review of records in the hospitals associated with the study mortuaries showed that death rates (deaths per 1000 admissions) in medical patients increased by 54% between 1983 and 1988, with increases of 106 and 98% in men aged 20 to 29 and 30 to 39 years, respectively, and 199 and 42% in women of the same age ranges. Over the same period, official Abidjan mortality statistics showed an increase in mortality rates (deaths per 100,000 per year) of 54% in men aged 20 years and older and of 28% in women aged 30 years and older. For discussion of possible demographic consequences of AIDS in developing countries, see R. M. Anderson, R. M. May, A. R. McLean, *Nature* **332**, 228 (1988).

16. We thank the Ministry of the Interior of the Ivory Coast for permission to examine vital statistics; the Ministry of Public Health and Population of the Ivory Coast; the heads of University Hospital Departments for access to records; the laboratory staff of Projet RETRO-CI; the United States Embassy, Abidjan, for logistic support; staff of the Division of HIV/AIDS, Centers of Disease Control (CDC); A. Nelson, Projet SIDA, Kinshasa, Zaire; R. L. Colebunders, Institute of Tropical Medicine, Antwerp, Belgium; J. Tafforeau, Free University of Brussels, Belgium; and M. Papaioanou, Division of HIV/AIDS, CDC, for discussion. See A. Nelson *et al.*, Fourth International Conference on AIDS, Stockholm, 12 to 16 June 1988 (abstract 5035).

30 January 1990; accepted 10 May 1990

Expression of Gene *rrg* Is Associated with Reversion of NIH 3T3 Transformed by LTR–c-H-*ras*

SARA CONTENTE, KAYLENE KENYON, DONATA RIMOLDI, ROBERT M. FRIEDMAN

A partial complementary DNA was isolated for a gene (*rrg*) that is normally expressed in mouse NIH 3T3 cells, but is down-regulated after cellular transformation by long terminal repeat (LTR)–activated c-H-*ras* (LTR–c-H-*ras*). This gene was reexpressed in a nontumorigenic persistent revertant cell line created by prolonged treatment of the transformed cells with mouse interferon α/β. Persistent revertants stably transfected with *rrg* complementary DNA antisense expression vectors appeared transformed, had decreased amounts of *rrg* messenger RNA, and were tumorigenic in nude mice. Stable transfection with sense constructs did not alter the normal morphology, message level, or nontumorigenicity of the persistent revertant cell line.

BOTH ACTIVATED *ras* ONCOGENES and elevated levels of normal *ras* product p21 have been found in a variety of human tumors (*1–4*). The inappropriate expression of the human cellular *ras* also leads to a transformed state in cultured mouse cells (*5*). Although there is a definite correlation between *ras* gene expression and cellular transformation, it is not yet clear by what means this oncogene exerts an effect on a cell's growth control system.

Samid *et al.* (*6–8*) examined the effect of interferon treatment on cell line RS485, a mouse NIH 3T3 cell line transformed by LTR–activated human c-H-*ras* (*5*). This treatment yielded persistently reverted cell lines, including PR4, that were phenotypically nontransformed and nontumorigenic, but maintained pre-reversion levels of *ras* mRNA and p21. It is possible that reversion by interferon involved either the activation or the deactivation of one or more genes that affect the transforming activity of *ras*. To identify such genes, we screened the transformed and persistent revertant cell lines for messages that were differentially expressed.

A cDNA phage library was prepared from the persistent revertant cell line PR4. Duplicate plaque lifts were hybridized with ^{32}P-labeled cDNA from PR4 and from the *ras*-transformed RS485. Plaques that exhibited differential hybridization signals were selected and rescreened until pure phage populations were obtained. The insert cDNAs of 14 recombinant phage were isolated, subcloned into the Eco RI site of plasmid vector pSP72, and used to probe RNA blots of NIH 3T3, RS485, and PR4. The expression of the "*ras* recision gene" (*rrg*, cDNA rrg-1) was dramatically reduced upon transformation of NIH 3T3 with LTR–c-H-*ras*, and was restored in the persistent revertant cell line (Fig. 1) to levels that varied among RNA preparations from 35 to 200% of

Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814-4799.

those in NIH 3T3. Treatment of RS485 with interferon α/β (1000 IU/ml) induced *rrg* expression after 7 to 10 days to one-tenth of the base-line level observed in NIH 3T3 (*9*). It is possible that only a fraction of the cell population responds to interferon. Six to 8 weeks of interferon treatment were required to derive the persistent revertant cell lines, and the reversion level was between 1 and 10% (*6, 7*). The rrg-1 cDNA hybridized with two mRNAs of approximately 4.5 and 6 kb. The larger message banded more diffusely and hybridized less strongly than the smaller message. Because rrg-1 was only about 700 bp in length, it was used to screen other cDNA libraries to obtain longer clones. A 3.4-kb cDNA (rrg-2) obtained from a murine B cell leukemia line cDNA library (*10*) gave the same hybridization pattern as rrg-1 on both RNA and DNA blots. Neither of these cDNAs contained a significant open reading frame, and the 3′ polyadenylated [3′ poly(A)] end of rrg-1 aligned with sequence 1.5 kb upstream of the 3′ poly(A) end of rrg-2. A probe derived from the 3′ 500 bp of rrg-2 hybridized only with the 6-kb message (*9*). Additional cDNAs of each size class that extended further 5′ were obtained from an NIH 3T3 cDNA library (rrg-4, 4.6 kb, and rrg-6, 3.1 kb). These cDNAs contained a 5′ open reading frame of at least 1000 bp. This sequence had no similarity to sequences in the GenBank (release 61) or the National Biomedical Research Foundation Data Bank (release 21) (*10a*). A full-length cDNA for *rrg*, which would contain an additional 1.5 kb at the 5′ end, has not yet been obtained.

We observed that *rrg* is conserved across some species (*9*). In human placenta, the rrg-2 cDNA hybridized with several Eco RI fragments superimposed over a smear; this mouse probe may hybridize with a human repetitive element. In the rat, rrg-2 hybridized with two Eco RI fragments of approximately 2 and 3 kb; rrg-2 did not appear to hybridize with DNA from mink or Syrian hamster.

Because the expression of *rrg* was restored during the process of persistent reversion, it is possible that a particular level of *rrg* expression is required to maintain this state. Antisense, or complementary, RNA is a useful tool for genetic analysis because it can inhibit specific gene expression in eukaryotes (*11–14*). A 3.5-kb Hind III fragment containing cDNA rrg-2 was subcloned downstream of the human β-actin promoter in the expression vector pHβAPr1-neo (*15*) in both the sense and antisense orientations. These constructs were each transfected (*16*) into cell line PR4 followed by selection with G418 (500 μg/ml). In five separate transfection experiments, a total of 108 G418^r sense colonies and 122 G418^r antisense colonies were obtained. Ten cell lines derived from the first four transfection experiments were chosen for further study. Each of the cell lines derived from transfection with rrg-2 in the sense orientation had a flat morphology similar to that of the PR4 cell line. The cell lines derived from transfection with rrg-2 in the antisense orientation showed morphologies varying from highly transformed (AS-3B and AS-30) to less flat, more refractile than PR4 (AS-4, AS-7, and AS-8).

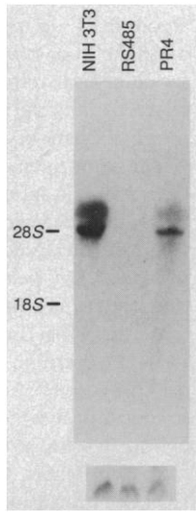

Fig. 1. RNA blot hybridized with rrg-1 cDNA. Total cell RNA (20 μg) from NIH 3T3, RS485, and PR4 was separated by electrophoresis on a 1% agarose–formaldehyde gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled rrg-1. Bottom panel shows hybridization with actin.

Table 1. Tumorigenicity of sense and antisense transfected cell lines. Cells (5×10^5 per site) were injected subcutaneously into 3-week-old female athymic mice (Division of Cancer Treatment, National Cancer Institute, Animal Program, Frederick Cancer Research Facility). Animals were examined daily for tumors. Results are presented as sites positive for tumor formation over sites injected. Tumors grew to ~1 cm in diameter within 7 days of first appearance (+++); within 7 to 14 days of first appearance (++); or 14 days or more after first appearance (+).

Cell line	Tumor growth	Day*	Tumors/ total sites
AS-3B	+++	10	4/4
AS-4	+	8	6/6
AS-7	++	17	4/6
AS-8			0/6
AS-30	+++	10	6/6
S-10			0/4
S-16			0/4
S-17			0/4
S-18		33	1/4
S-27	++	26	4/4
S-27	++	15	3/6
PR4-neo^r			0/4
RS485	+++	8	4/4

*Day of first appearance of tumor.

Integrated rrg-2 expression plasmid was present in both the sense and antisense cell lines (Fig. 2). The strongly hybridizing 4.4-kb Hind III fragment derives from the endogenous *rrg*. There are about ten copies of *rrg* per diploid genome. Each of the cell lines also contained other fragments of varied size that hybridized with rrg-2, indicating that the plasmid construct was integrated into the cellular DNA [S-16 DNA was not loaded on the gel in Fig. 2; however, this line does contain integrated sense construct (*17*)]. The number of copies of plasmid integrated into cellular DNA varied from one (line AS-4) to fifty (line AS-8).

The expression level of the *rrg* messages (Fig. 3) in the sense cell lines was comparable to that seen in NIH 3T3 and PR4, whereas the expression level of the *rrg* messages in the antisense cell lines was markedly reduced.

The tumorigenicity of the sense and antisense transfected cell lines was tested by injecting them subcutaneously into athymic mice (Table 1). Of the five antisense cell lines tested, only one line (AS-8) failed to induce tumor formation. This cell line grew

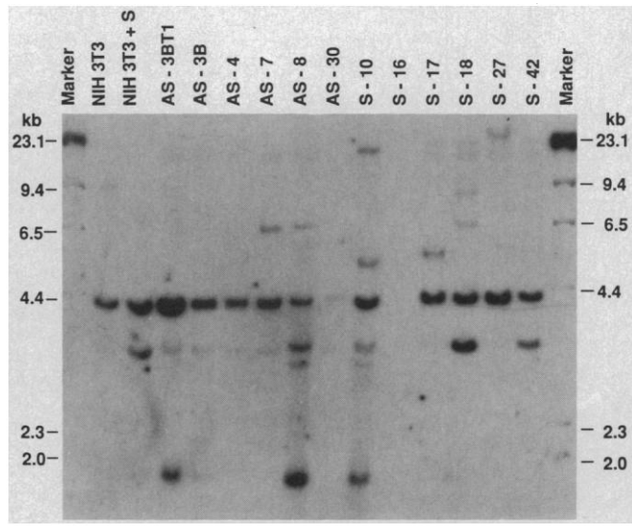

Fig. 2. High molecular weight DNA blot of sense and antisense cell lines, hybridized with rrg-2. High molecular weight DNA (30 μg) isolated from each cell line, was digested with Hind III, separated by electrophoresis on a 1% agarose gel, and transferred to a nylon membrane. Marker, λ–Hind III; S, sense; AS, antisense; AS-3BT1, cell line cultured from tumor induced in a nude mouse by subcutaneous injection of cell line AS-3B; NIH 3T3 + S, 30 μg of NIH 3T3 DNA mixed with 0.45 ng of sense expression plasmid. DNA from S-16 was not loaded on gel.

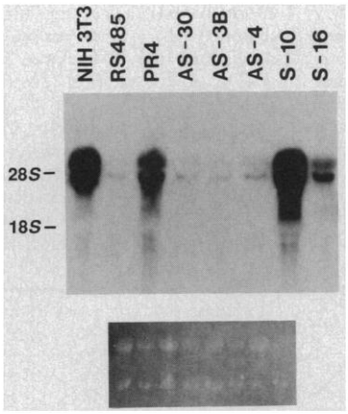

Fig. 3. RNA blot of sense and antisense cell lines hybridized with rrg-1. Total cellular RNA (20 μg) was separated by electrophoresis on a 1% agarose–formaldehyde gel and transferred to a nylon membrane. S, sense; AS, antisense. Autoradiogram was overexposed to show AS lanes. Bottom panel shows ethidium bromide staining of 28*S* and 18*S* RNA.

poorly in culture and was lost before RNA analysis could be completed, although DNA analysis showed that it contained integrated expression construct (Fig. 2). The cells from antisense lines that induced tumor formation did so within the same time frame as cells from the transformed line RS485, although some antisense lines were more tumorigenic than others.

Four of the five sense cell lines tested were nontumorigenic after 8 weeks of observation, along with the control cell line PR4 that had been transfected with the pHβAPr1-neo vector. The sense line that was tumorigenic (S-27) had a normal morphology and contained amounts of *rrg* mRNA comparable to those seen in the other sense cell lines. However, a cell line cultured from a tumor induced by S-27 appeared transformed and had very low amounts of *rrg* mRNA. The S-27 cell line most likely contained a very small number of spontaneously re-transformed cells that were selected out in the athymic mice, resulting in tumor formation.

The tumorigenicity of the four antisense lines does not appear to be due to spontaneous re-transformation of the revertant cells after DNA transfection, since the sense construct did not yield a similar proportion of tumorigenic cell lines.

We have been unable as yet to demonstrate the presence of antisense transcripts in the antisense cell lines. Antisense RNA transcription probes generated in vitro did not hybridize with blots of total cellular RNA from antisense cell lines; ribonuclease (RNase) protection and nuclear run-on experiments have also been inconclusive. Similar difficulties in demonstrating the presence of antisense transcripts in eukaryotic systems have been noted by other investigators (*18, 19*).

A marked decrease in *rrg* expression was also observed in other *ras*-transformed cell lines (Fig. 4). NIH 3T3 transformed by the mutated EJ *ras* (*20*) or by multiple copies of the normal c-H-*ras* (line 136) (*21*), as well as Balb/c cells containing v-K-*ras* and v-H-*ras* (*22*), all showed reduced *rrg* expression. The cell line DT, containing two copies of v-K-*ras* (*23*), constituted an exception. Among other oncogene-transformed NIH 3T3 cells, v-*raf* (*24*) and v-*fes* (*25*) transformants also exhibited very low *rrg* transcript levels, whereas variable amounts of message were found in cells with c-*myc* (*26*) controlled by SV40 early promoter, v-*abl* (*27*), v-*sis* (*28*), and v-*mos* (*29*).

Our data suggest that cellular transformation induced by *ras* requires down-regulation of the *rrg* gene product. The expression of *rrg* was dramatically decreased upon cellular transformation by *ras* and was restored during interferon-mediated reversion, leading to a nontransformed, nontumorigenic state in cells that still had a high level of *ras* expression. The findings with the antisense transfection studies suggest that *rrg* expression alone is sufficient to revert the *ras*-transformed RS485. Transfection of NIH 3T3 with the antisense construct does not in itself lead to cellular transformation (*9*), indicating that down-regulation of *rrg* is not sufficient for cellular transformation.

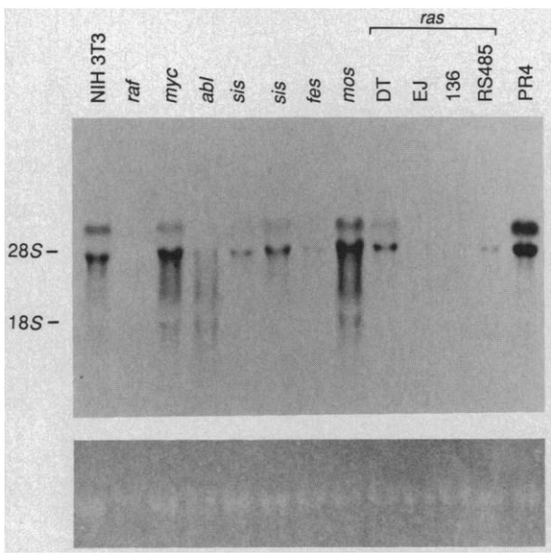

Fig. 4. RNA blot of oncogene-transformed NIH 3T3 cell lines hybridized with rrg-1 cDNA. Total cell RNA (25 μg) was separated by electrophoresis on a 1% agarose–formaldehyde gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled rrg-1. Bottom panel shows ethidium bromide staining of 28*S* RNA.

REFERENCES AND NOTES

1. J. L. Bos, *Cancer Res.* **49**, 4682 (1989).
2. P. H. Hand, V. Vilasi, A. Thor, N. Ohuchi, J. Schlom, *J. Natl. Cancer Inst.* **9**, 59 (1987).
3. D. A. Spandidos and I. B. Kerr, *Br. J. Cancer* **49**, 681 (1984).
4. T. Tanaka, D. J. Slamon, H. Battifora, M. J. Cline, *Cancer Res.* **46**, 1465 (1986).
5. E. H. Chang, M. E. Furth, E. M. Scolnick, D. R. Lowy, *Nature* **297**, 479 (1982).
6. D. Samid, E. H. Chang, R. M. Friedman, *Biochem. Biophys. Res. Commun.* **119**, 21 (1984).
7. ———, *ibid.* **126**, 509 (1985).
8. D. Samid, D. M. Flessate, R. M. Friedman, *Mol. Cell. Biol.* **7**, 2196 (1987).
9. S. Contente, K. Kenyon, R. M. Friedman, unpublished data.
10. Y. Ben-Neriah, A. Bernards, M. Paskind, G. Q. Daley, D. Baltimore, *Cell* **44**, 577 (1986).
10a. The sequence of rrg-1 has been deposited with GenBank, accession number M35796.
11. J. G. Izant and H. Weintraub, *ibid.* **36**, 1007 (1984).
12. ———, *Science* **229**, 345 (1985).
13. J. P. Green, O. Pines, M. Innouge, *Annu. Rev. Biochem.* **55**, 569 (1986).
14. H. Weintraub, J. G. Izant, R. M. Harland, *Trends Genet.* **1**, 22 (1985).
15. P. Gunning, J. Leavitt, G. Muscat, S. Y. Ng, L. Kedes, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4831 (1987).
16. C. A. Chen and H. Okayama, *BioTechniques* **6**, 632 (1988).
17. S. Contente, K. Kenyon, R. M. Friedman, unpublished data.
18. U. Kasid *et al.*, *Science* **243**, 1354 (1989).
19. R. Khokha *et al.*, *ibid.*, p. 947.
20. C. Shih and R. A. Weinberg, *Cell* **29**, 161 (1982).
21. S. Pulciani, E. Santos, L. K. King, V. Sorrentino, M. Barbacid, *Mol. Cell. Biol.* **5**, 2836 (1985).
22. S. Contente, K. Kenyon, R. M. Friedman, unpublished data.
23. M. Noda, Z. Selinger, E. M. Scolnick, R. H. Bassin, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5602 (1983).
24. H. W. Jansen *et al.*, *Nature* **307**, 281 (1984).
25. T. Hanafusa *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3009 (1980).
26. H. Land, L. Parada, R. A. Weinberg, *Nature* **304**, 596 (1983).
27. P. L. Green, D. A. Kaehler, R. Risser, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5932 (1987).
28. K. C. Robbins, S. G. Devare, E. P. Reddy, S. A. Aaronson, *Science* **218**, 1131 (1982).
29. D. G. Blair, M. Oskarsson, W. L. McClements, G. F. Vande Woude, *Hamatol. Bluttransfus.* **26**, 460 (1981).

30. We thank E. Chang for valuable discussions and for providing the NIH 3T3 cDNA library. Thanks to H. Young (K- and H-Balb/c), D. Blair (M-MuLV), R. Risser (psi2 line 1806-6), R. Bassin (DT), and M. Barbacid (136). We also thank C. Cohn, E. Weidenhammer, and C. Kubrak for technical assistance. Supported by NIH grant R01 CA 37351-04A1 and an agreement with the National Foundation for Cancer Research.

19 December 1989; accepted 12 June 1990