In hibernating animals, the maximum contractile force of ventricular muscle was obtained at the lowest driving frequency (0.05 Hz), an indication that this is favorable for hibernating animals which have slow heart beats. In addition, dV/dt_{max} was significantly greater in hibernating animals than in nonhibernating animals (Table 1); the physiological significance of this difference is unclear.

Caffeine augments calcium influx during the action potential and simultaneously reduces the contribution of intracellular calcium stores to myocardial contraction (*6, 7*). Treatment with caffeine (5 m*M*) caused the positive inotropic effect (210 ± 38 percent of control; $n = 5$) with an increase in APD 50 in nonhibernating animal preparations driven by low frequency (0.2 Hz), whereas it caused the negative inotropic effect in hibernating animals (56.5 ± 6.5 percent of control; $n = 5$) in spite of an increase in APD 50 (Fig. 2, A and B). In addition, ryanodine (2×10^{-6} *M*), an inhibitor of the release of calcium from sarcoplasmic reticulum (*8*), abolished the developed tension of ventricular muscle from the hibernating animals but partly inhibited cardiac contraction in the nonhibernating animal preparation (55.8 ± 4.6 percent of control; $n = 6$). These differences in the effects of caffeine and ryanodine on the two preparations further suggest that intracellularly derived calcium makes a greater contribution to the activation of contraction in the myocardium of hibernating animals.

In summary, the electrical and mechanical characteristics of heart muscle are different in hibernating and nonhibernating animals; hibernation may cause a change in the excitation-contraction coupling mechanisms of myocardium. The possible mechanism of contraction during hibernation by which a major part of activator calcium is intracellularly derived is considered to be suitable for effective myocardial function at low temperatures. One might ask whether this change is a direct or an indirect result of hibernation. However, since the intracellular *p*H of the heart is not changed during hibernation (*9*), and prolonged cold exposure does not affect the electrical characteristics of mammalian myocardium (*10*), some possibilities for an indirect influence of hibernation can be eliminated. Although the precise explanation of this mechanism is not clear, it is interesting that in one animal myocardial properties can be exchanged in accordance with environmental changes. These findings can provide a model in which the physiology of the heart or drug effects on different calcium pools can be studied in otherwise similar preparations and may open a new pathway to understanding the mechanism of cold tolerance in hibernating animal myocardium.

NORIAKI KONDO*
Department of Pharmacology, Mitsubishi-Kasei Institute of Life Sciences, Machida, Tokyo 194, Japan
SHOJI SHIBATA
Department of Pharmacology, University of Hawaii, Honolulu, Hawaii 96844

References and Notes

1. C. P. Lyman and P. Chatfield, *Physiol. Rev.* **35**, 403 (1955); in *Handbook of Physiology*, M. B. Visscher, A. B. Hasting, J. R. Pappenheimer, H. Rhan, Eds. (Waverly, Baltimore, 1965), p. 1967.
2. A. R. Dawe and P. R. Morrison, *Am. Heart J.* **49**, 367 (1955); C. P. Lyman and D. C. Blinks, *J. Cell. Comp. Physiol.* **54**, 53 (1959); D. E. Smith and B. Katzung, *Am. Heart J.* **71**, 515 (1966); J. B. Senturia, S. Stewart, M. Menaker, *Comp. Biochem. Physiol.* **33**, 43 (1970).
3. W. Trautwein, *Physiol. Rev.* **53**, 793 (1973); H. Irisawa, *ibid.* **58**, 461 (1978).
4. P. F. Cranefield, *The Slow Response and Cardiac Arrhythmias* (Futura, New York, 1975).
5. K. A. Edman and M. Johanson, *J. Physiol. (London)* **228**, 259 (1976); A. Fabiato and F. Fabiato, *Ann. N.Y. Acad. Sci.* **307**, 491 (1978).
6. R. Borden and K. H. Sonnenblick, *Am. J. Physiol.* **228**, 259 (1975); A. H. Henderson, D. L. Brutsaert, R. Forman, E. M. Sonnenblick, *Cardiovasc. Res.* **8**, 162 (1974).
7. J. R. Blinks, C. B. Olson, B. R. Jewell, P. Braveny, *Circ. Res.* **30**, 367 (1972); D. R. Hunter, R. A. Haworth, H. A. Berkoff, *ibid.* **51**, 363 (1982); H. Jundt, H. Porzig, H. Reuter, J. W. Stucki, *J. Physiol. (London)* **246**, 229 (1975).
8. D. J. Jenden and A. S. Fairhurst, *Pharmacol. Rev.* **21**, 1 (1969); L. R. Jones, H. R. Besh Jr., J. L. Sutko, J. T. Willerson, *J. Pharmacol. Exp. Ther.* **209**, 48 (1979); J. L. Sutko and J. T. Willerson, *Circ. Res.* **46**, 332 (1980).
9. A. Malan, *Experientia* (Suppl.) **32**, 303 (1978).
10. K. Matsuda, T. Hoshi, S. Kameyama, H. Kusakari, K. Sakurai, *Seitainokagaku* **9**, 21 (1958).

* To whom correspondence should be addressed.

17 October 1983; accepted 1 June 1984

An Activated *ras*^{N} Gene: Detected in Late But Not Early Passage Human PA1 Teratocarcinoma Cells

Abstract. *Early passages of the human teratocarcinoma cell line PA1 are not tumorigenic in nude mice, while late passages are. A transforming gene present in late passages of PA1 cells was isolated as a biologically active molecular clone and is a new isolate of the human* ras^N *locus. Its transforming activity is due to a single* $G \rightarrow A$ *(G, guanine; A, adenine) point mutation at the codon for amino acid 12 which changes the codon for glycine so that an aspartic acid residue is expressed. In contrast to late passage PA1 cells (passages 106, 330, and 338), DNA from the PA1 cell line at early passages (passage 36) does not yield* ras^N *foci in DNA transfection assays. Thus, the presence of an activated* ras^N *in PA1 cells correlates with enhanced tumorigenicity of the cell line and, more importantly, may have arisen during cell culture in vitro.*

Experiments with viral DNA preparations in which gene transfer techniques were used (*1*) were the first to demonstrate DNA-mediated cell transformation in cultures of animal cells. Shih *et al.* (*2*) used this methodology to show that DNA preparations from transformed mouse cells could transfer that phenotype to recipient cultures of NIH 3T3 mouse cells. These findings have led investigators to invoke a genetic theory for cell transformation based on one or more genetic changes, giving rise to a transforming gene that can be assayed in the DNA-mediated transformation of NIH 3T3 cells. It may be that these transforming genes are involved in the multistep progression which ultimately gives rise to the tumorigenic phenotype.

Transforming genes have been detected in the NIH 3T3 cell assay in DNA preparations from various tumors and tumor cell lines (*3*). A number of the transforming genes molecularly cloned from human tumors and tumor cell lines have been shown to be cellular homologs of the oncogenes of the Harvey (*ras*^{H}) and Kirsten (*ras*^{K}) sarcoma viruses (*4–9*). An additional *ras*-related cellular genetic locus, *ras*^{N}, has been identified by transfer to NIH 3T3 cells with the use of DNA from the SK-N-SH neuroblastoma cell line (*10*). Uniformly, the difference responsible for activation of *ras* genes between normal or tumor sources has been found to be point mutations resulting in coding sequence changes for the *ras* protein (p21) (*11–13*).

We have described (*14*) the detection of a transforming gene in DNA isolated from a human teratocarcinoma cell line, PA1 (*15*), at passage 330. The cell line was derived from the culturing of ascites fluid cells from a patient with a metastatic ovarian germ-line tumor and has properties of embryonal carcinoma cell lines. The cells are capable of differentiation in culture under adherent or nonadherent conditions. The cell line shows significant alterations in various malignancy-related phenotypes with extended passage in culture (*15*). Early passage PA1 cells grow slowly in culture and form tumors in athymic nude mice only after

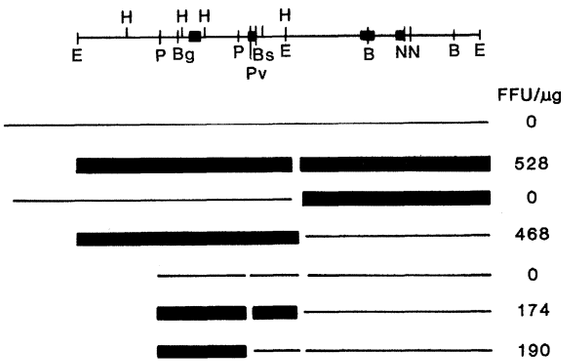

Fig. 1. Activation analysis of the PA1 *ras*^{N} clone. (Top) Restriction map of cloned *ras*^{N} oncogene in plasmid pMTJ. Symbols: E, Eco RI; H, Hind III; P, Pst I; Pv, Pvu; Bg, Bgl II; Bs, Bst EII; N, Nde I; B, Bam HI. (Bottom) Restriction fragments of the PA1 *ras*^{N} clones or human placental *ras*^{N} clones were isolated by agarose gel electrophoresis and ligated at the junctions indicated by gaps in the lines. The biological activity in focus-forming units per microgram (FFU/μg) were carried out as described (*19*). Thin lines indicate sequences derived from placental *ras*^{N} clones. Wide lines indicate sequences derived from PA1 *ras*^{N} clones.

long latent periods of 17 to 30 weeks (*15*). In contrast, late passage PA1 cells grow rapidly in culture and readily form tumors in athymic nude mice in 7 to 11 weeks. In this study, we correlate the presence of an activated *ras* oncogene with the increased level of tumorigenicity observed in late cell culture passages 106, 330, and 338.

We have studied the nature of the human DNA sequences from passage 330 PA1 cells ($PA1_{330}$) present in secondary NIH 3T3 foci by Southern blot analysis (*14*). No sequences related to oncogenes of the Harvey or Kirsten sarcoma viruses were detected other than those present in the normal mouse genome. However, a human *ras*^{N} probe revealed related sequences in DNA from PA1 secondary foci. A representative genomic library was constructed from DNA obtained from a $PA1_{330}$ secondary focus partially digested with the restriction enzyme Sau 3A I with the use of the bacteriophage vector EMBL-3 (*16*). This genomic library was screened with a *ras*^{N} probe and phage clones were isolated which contained this activated *ras*^{N} on two Eco RI fragments each 7.0 kb long. The normal *ras*^{N} locus contains a 9.2-kb Eco RI fragment carrying the first and second exons and a 7.0-kb fragment carrying the third and fourth exons. The cloned *ras*^{N} from the $PA1_{330}$ tranformed secondary focus has the Eco RI fragment rearranged 5′ to the first exon of *ras*^{N} and this Eco RI fragment is truncated to 7.0 kb. Shimizu *et al.* have a similar result for the first *ras*^{N} isolate (*17*). From a comparison with the *ras*^{N} restriction map, we concluded that the biologically active gene could be obtained by ligation in the proper orientation of the above Eco RI 7.0-kb fragment with another 7.0-kb fragment containing the 3′ portion of the gene. Therefore, those two Eco RI fragments were cloned onto the plasmid vector pBR322 to reassemble the gene. This plasmid clone, pMTJ, was assayed for biological activity in the NIH 3T3 DNA-mediated transformation assay. This construct was found to efficiently transform NIH 3T3 cells (3×10^3 to 8×10^3 focus-forming units per microgram).

Since other human *ras* genes have been found to be activated at either amino acid position 12 or 61 (*11–13, 18–22*), we proceeded to analyze the DNA sequence of the first two exons of the transforming gene from the human teratocarcinoma $PA1_{330}$. The DNA sequence of the second exon exactly matched that of the normal human *ras*^{N} (*23*). However, DNA sequence of the first exon revealed a change in a single nucleotide base corresponding to codon for amino acid 12 (data not shown). Instead of GGT (G, guanine; T, thymine) coding for the amino acid glycine, the sequence GAT (A, adenine) was found, corresponding to a change to aspartic acid. This therefore demonstrates that *ras*^{N}, like *ras*^{K} and *ras*^{H}, can be activated by point mutation at the codon for position 12. Previously, only a change at amino acid 61 had been reported for *ras*^{N} but the human *ras*^{H} has been found to be activated at either position 12 or position 61 (*22*).

In order to verify that the mutation giving rise to a change in amino acid position 12 is responsible for activation of these genes, we performed a transformation experiment. The first exon of the $PA1_{330}$-transforming gene resides on a Pst I fragment. This fragment was ligated to the Pst I–Eco RI fragment containing the second exon of the normal *ras*^{N} and subsequently to the 7.0-kb Eco RI fragment containing the third and fourth exons of the normal *ras*^{N}. This combination as well as control permutations was transfected onto NIH 3T3 cells (Fig. 1). Only the Pst I fragment containing the first exon of the *ras*^{N} PA1 transforming gene is able to activate the normal sequences in the remaining three exons to generate a biologically active transforming gene. Thus, we concluded that the mutation at the codon for amino acid 12 is responsible for the activation of this *ras*^{N} isolate.

The low tumorigenicity reported for early passage PA1 cells (*15*) prompted us to investigate whether the activated *ras*^{N} gene was present in early (passage 36) and late (106 and 338) passages. As previously reported (*15*) late passage PA1 cells are tumorigenic in nude mice (Table 1). However, although Zeuthen *et al.* (*15*) reported low tumorigenicity for early passage PA1 cells, our early passage PA1 cells (passage 36) did not form tumors in nude mice, even at the high cell density after 24 weeks (Table 1).

We compared the transforming activity of DNA isolated from early and late passage PA1 cells in both the NIH 3T3

Table 1. Tumor formation in athymic nude mice. PA1 cells were grown to 80 percent confluency, trypsinized, and inoculated subcutaneously into nude mice. Early and late passage PA1 cells were identical by alloenzyme analysis.

Passage	Cells inoculated	Tumor (No.)*	Latency (weeks)
36	10^6	0/4	>24
	10^7	0/3	>24
106	10^6	2/3	11
338	10^6	4/4	7
	10^7	3/3	5

*Ratio of number of tumors to number of animals tested.

Table 2. DNA-mediated transformation into NIH 3T3 cells. Comparison of early and late PA1 cells for presence of activated *ras*^{N} DNA-mediated transformation analysis by direct focus formation was carried out as described (*14*). Transfection followed by tumor formation analysis was carried out by transfecting 25 μg of cell DNA plus 2.0 μg of pSV2 neo-DNA per 5×10^5 NIH 3T3 cells in a 60-mm culture dish. Twenty-four hours later the cells from two dishes were trypsinized and seeded into a T-150 culture flask. Twenty-four hours later the antibiotic G418 was added to the medium at 400 μg/ml. At 14 days after transfection, the transfected cells were trypsinized, counted, and inoculated into nude mice at 1×10^6 to 3×10^6 cells per mouse. The animals were examined weekly for tumors.

Passage	Tissue culture focus formation (foci per plate)	Tumor formation	
		Animals	Latency (weeks)
	PA1 DNA		
37	0/12	4/16*	7
36	0/4		
106	2/8		
330	4/8		
338	7/12	14/18	4
	Human placental DNA		
	1/16	0/10	

*DNA from these tumors are free of human *ras*^{N} sequences.

cell DNA-mediated transfection and nude mouse transformation assays (*24*) (Table 2). In the transfection assay no foci were observed with DNA from early passage PA1 cells under conditions that gave foci from late passage (106, 330, and 338) cell DNA. Moreover, like $PA1_{330}$-transformed NIH 3T3 cells, cells transformed with DNA from passage 106 and 338 PA1 cells have acquired the human ras^N sequences.

In the nude mouse transformation assay (*24*) DNA from early passage PA1 cells, late passage PA1 cells or human placenta was first co-transfected onto NIH 3T3 cells with the neomycin resistant plasmid pSV2-neo (*25*). Cells were then cultured for 2 weeks under the selective pressure of the antibiotic G418 prior to inoculation into nude mice. Of 18 nude mice, 14 developed tumors within 4 weeks after inoculation of cells receiving a late passage (338) DNA (Table 2). In contrast to the lack of focus-forming activity with the early passage cell DNA, tumors did appear in the nude mice receiving cells transfected with early passage PA1 DNA after 7 weeks; however, DNA from these tumors do not contain human ras^N DNA sequences. These analyses suggest that the early passage PA1 cells do not have activated ras^N as compared to late passage PA1 cells. This may be related to the inability of the early passage PA1 cells to clone in soft agar (data not shown) or their lack of tumorigenicity in nude mice (Table 1). Since passage 106 and 338 cells form colonies in soft agar and are tumorigenic in athymic nude mice, we can correlate tumorigenicity with the presence of the activated ras^N.

In addition to the activated ras^N, there are at least two other genetic changes occurring in PA1 cells. One is the "non-ras^N" dominant transforming gene in $PA1_{37}$ cells as observed in the occurrence of tumors in the nude mouse DNA transfection assay (Table 2). The second is a balanced translocation between chromosomes 15 and 20. Zeuthen *et al.* (*15*) have reported that this translocation was present at passage 224 but absent from passage 24 cells. We have observed this translocation in passage 45 and 338 PA1 cells. These genetic changes precede the appearance of the ras^N mutation in PA1 cells and may predispose the cell to selection of the activated ras^N.

There are few explanations for the appearance of the activated ras^N gene in late passage PA1 cells. It is possible that cells containing a mutant ras^N gene may have been present at low levels in the original ascites fluid. The patient with the teratocarcinoma was given chemotherapy before isolation of the ascites fluid, and it is conceivable that such a treatment may have preferentially removed cells containing the activated ras^N. However, if the activated ras^N is sufficient to provide a selective growth advantage, those few cells in the original population would be expected to achieve predominance in the culture by passage 37. Therefore, if the mutant ras^N is sufficient to provide the selective advantage, it is more reasonable that the activation arose during passage in culture.

MICHAEL A. TAINSKY
Laboratory of Molecular Oncology, National Cancer Institute, Frederick Cancer Research Facility, Frederick, Maryland 21701
COLIN S. COOPER
Laboratory of Molecular Carcinogenesis, Dana-Farber Cancer Institute, Boston, Massachusetts 02115
B. C. GIOVANELLA
Stehlin Foundation for Cancer Research, Houston, Texas 77002
GEORGE F. VANDE WOUDE
LBI-Basic Research Program, Frederick Cancer Research Facility

References and Notes

1. F. L. Graham and A. J. Vander Eb, *Virology* **52**, 456 (1973).
2. C. Shih, B. Z. Shelo, M. P. Goldfarb, A. Dannenberg, R. A. Weinberg, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5714 (1979).
3. M. A. Lane, A. Sainter, G. M. Cooper, *Cell* **28**, 873 (1982).
4. L. F. Parada, C. J. Tabin, C. Shih, R. A. Weinberg, *Nature (London)* **297**, 474 (1982).
5. E. Santos, S. R. Tronick, S. A. Aaronson, S. Pulciani, M. Barbacid, *ibid.* **298**, 343 (1982).
6. C. J. Der, T. G. Krontris, G. M. Cooper, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3637 (1982).
7. C. J. Der and G. M. Cooper, *Cell* **32**, 201 (1983).
8. M. P. Goldfarb, K. Shimizu, M. Perucho, M. H. Wigler, *Nature (London)* **296**, 404 (1982).
9. A. Hall, C. J. Marshall, N. K. Spurr, R. A. Weiss, *ibid.* **303**, 396 (1983).
10. K. Shimizu, M. Goldfarb, M. Perucho, M. Wigler, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 383 (1983).
11. E. P. Reddy, R. K. Reynolds, E. Santos, M. Barbacid, *Nature (London)* **300**, 149 (1982).
12. C. J. Tabin *et al.*, *ibid.*, p. 143.
13. E. Taparowsky, Y. Suard, O. Fasano, K. Shimizu, M. Goldfarb, M. Wigler, *ibid.*, p. 762.
14. C. S. Cooper, D. G. Blair, M. K. Oskarsson, M. A. Tainsky, L. A. Eader, G. F. Vande Woude, *Cancer Res.* **44**, 1 (1984).
15. J. Zeuthen, J. D. R. Norgaard, P. Avner, M. Fellows, J. Wartiovaara, A. Vaheri, J. Rosen, B. C. Giovanella, *Int. J. Cancer* **25**, 19 (1980).
16. A. Frishaul, H. Lehrach, A. Poustka, N. Murray, *J. Mol. Biol.* **170**, 827 (1983).
17. E. M. Southern, *ibid.* **98**, 503 (1975).
18. K. Shimizu *et al.*, *Nature (London)* **304**, 507 (1983).
19. D. Capon *et al.*, *ibid.*, p. 507.
20. N. Tsuchida, T. Ryder, E. Ohtsubo, *Science* **217**, 937 (1982).
21. R. Dhar *et al.*, *ibid.*, p. 934.
22. Y. Yuasa, S. K. Scrivastava, C. Y. Dunn, J. S. Rhim, E. P. Reddy, S. A. Aaronson, *Nature (London)* **303**, 775 (1983).
23. E. Taparowsky, K. Shimza, M. Goldfarb, M. Wigler, *Cell* **34**, 586 (1983).
24. D. G. Blair, C. S. Cooper, M. K. Oskarsson, L. A. Eader, G. F. Vande Woude, *Science* **218**, 1122 (1982).
25. P. Berg and P. J. Southern, *Mol. Appl. Genet.* **1**, 327 (1982).
26. We thank M. Wigler and E. Taparowsky for ras^N clones and data prior to publication, F. Shamanski for technical assistance, F. Propst for advice in DNA sequence analysis, W. Peterson for karyotype analysis and alloenzyme analysis, and a reviewer for useful comments.

9 March 1984; accepted 22 May 1984

New Radiocarbon Dates on the Cereals from Wadi Kubbaniya

In 1978, three carbonized grains of barley and a carbonized grain of einkorn wheat were found in a buried hearth at a Late Paleolithic site (E-78-4) at Wadi Kubbaniya in Egypt (*1, 2*). Another grain of barley was found elsewhere on the same buried living floor. In 1981, two large clusters of barley seeds, which were identified as six-row barley and thus domestic, were found at a nearby site (E-78-3) of comparable age (*3*). Numerous grinding stones, presumed to have been used for processing the cereals, were found in these and other sites, often deeply buried, and 30 radiocarbon dates placed the occupations between 18,500 and 17,000 radiocarbon years ago. These finds led us to suggest an early origin of food production, with implications for the initial development of complex societies (*4*).

Several barley seeds from site E-78-3 were analyzed by electron spin resonance spectroscopy to determine the maximal temperature to which they had been subjected before burial. A temperature of only some 150°C was indicated (*5*), which is too low to have brought about the charring required for them to have survived millennia of seasonal flooding.

Therefore, six barley seeds and three small pieces of wood charcoal were dated directly by using a tandem accelerator mass spectrometer. One piece of charcoal was from site E-78-3, from a layer just above the two clusters of seeds found in 1981; the other two came from site E-78-4, from the same living-floor as the cereals. One seed was from each cluster at E-78-3, three from the group in the hearth at E-78-4, all of which had been coated with gold for scanning electron microscopy (SEM) (*6*), and the last, found a short distance from the hearth, had not been coated.

To make the tandem accelerator measurements shown in Table 1, the seeds were converted to carbon powder and mixed with pure iron powder. This mixture was melted to form iron-carbon beads, which were mounted on the ion source of the accelerator. The ratios of $^{14}C/^{13}C$ in the beads and in standard samples of iron carbide made from A.D. 1890 tree-rings were made in a manner