prevalent in sylvan animals [for example, over 25 percent of white-tailed deer in the southeastern United States circulate microfilariae of *Setaria yehi* (*13*)]. Because microfilaremias are of long duration in natural vertebrate hosts, concurrent infections should not be uncommon in nature. We suggest that filarial infection may promote transmission of arboviral infection in nature.

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6. In each experiment, paired gerbils (one microfilaremic and one of similar weight but not infected by *B. malayi*) were each inoculated intraperitoneally with $10^{7.0}$ plaque-forming units of virus. One day later, each gerbil was anesthetized with sodium pentobarbital and placed in a cage containing about 250 5- to 8-day-old female *Ae. taeniorhynchus* (Vero Beach strain). The mosquitoes were allowed to feed for 15 minutes. To determine quantity of virus ingested, five freshly engorged mosquitoes were removed from each cage, immediately triturated individually in 1 ml of diluent, and then frozen at −70°C for later analysis by plaque assay for infectious virus in Vero cells [T. P. Gargan, C. L. Bailey, G. A. Higbee, A. Gad, S. El Said, *Am. J. Trop. Med. Hyg.* **32**, 1154 (1983)]. Diluent consisted of Medium 199 with Hanks salts containing 10 percent calf serum, 0.075 percent $NaHCO_3$, and antibiotics [penicillin (100 unit/ml), streptomycin (100 μg/ml), gentamycin (50 μg/ml), and fungizone (5 μg/ml)]. The number of microfilariae ingested was determined from the midgut of ten freshly engorged mosquitoes. The remaining engorged mosquitoes were placed in 3.8-liter cardboard containers with netting over one end and maintained at 26°C and 16 hours of light per day. Apple slices or a 10 percent sucrose solution were provided as nutriment. An oviposition dish was placed in each cage 3 days later, and mosquitoes were removed from each cage for vector competence studies beginning the next day. Transmission trials were conducted by permitting mosquitoes to feed either individually or in pairs on restrained hamsters (T. P. Gargan *et al.*, *ibid.*). After the feeding attempt, each mosquito was chilled and its legs and body were triturated separately in 1 ml of diluent. Presence of virus in legs provided evidence of a disseminated infection. Each mosquito was also examined for evidence of feeding on the hamster. Because RVF viral infection is virtually 100 percent fatal to hamsters (T. P. Gargan *et al.*, *ibid.*), hamster death was used as the criterion for virus transmission. A 20 percent liver suspension from a sample of the dead hamsters was assayed for virus content. Virus identity was confirmed by a standard neutralization test. Virus transmission rate was defined as the number of mosquitoes that refed and transmitted virus divided by the number that refed, regardless of their infection status.
7. The Zagazig Hospital 501 strain of RVF virus was used throughout this study after two passages in fetal rhesus monkey lung cells.
8. Male gerbils were infected with *B. malayi* by subcutaneous inoculation of 200 third-stage larvae 8 months before their use in these experiments.
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Isolation, Characterization, and Chromosome Assignment of Mouse N-*ras* Gene from Carcinogen-Induced Thymic Lymphoma

Abstract. *Treatment of mice with the carcinogen* N-*methylnitrosourea results in the development of thymic lymphomas with frequent involvement of the N*-ras *oncogene. The activated mouse N*-ras *gene was isolated from one of these lymphomas and, by transformation in concert with restriction digestion, a map of the gene was prepared and its approximate boundaries were determined. By means of somatic cell hybrids the normal N*-ras *gene was found to be unlinked to other members of the* ras *gene family.*

DNA from several human tumors and tumor cell lines contains oncogenes that can transform NIH 3T3 cells (*1*). Oncogenes can be classified into two functional groups known as the *lym* (*2*) and *ras* (*3*) families. Three members of the *ras* family have been identified: H-, K-, and N-*ras*. The N-*ras* gene is the only member of the *ras* family that has not been found in RNA viruses. Animal models are available in which *ras* genes are associated with tumor development (*4*). When working with a mouse model, we found that treatment with the chemical carcinogen *N*-methylnitrosourea (NMU) or with γ-radiation causes the formation of thymic lymphomas, the DNA of which induces foci in rodent fibroblasts (*4*). We identified the activated oncogenes in these tumors as *ras* family members because the transforming phenotype segregated with extra copies of these oncogenes in isolated foci (*4*).

The activated oncogene was isolated from a rat secondary transformant obtained with DNA originally derived from an NMU-induced mouse thymic lymphoma (the 3T3 primary transformant and this rat secondary transformant were both tumorigenic in nude mice). Use of the rat secondary instead of the NIH 3T3 primary transformant allowed us to distinguish the active oncogene from endogenous homologs, because of the species differences.

First we studied the effect of Eco RI on the transforming activity of the gene and found that this enzyme destroyed its activity (Table 1). It was therefore necessary to isolate a partial product of higher molecular weight in order to obtain a functional gene. We also needed a strategy to distinguish the mouse N-*ras* gene from any rat genes with similar sequences.

When genomic DNA from rat cells and from the secondary transformant was partially digested with Eco RI and sub-

Table 1. Transforming activity of the oncogene present in carcinogen-induced mouse thymic lymphoma. Transformations were performed as described (*17*) with 40 μg of genomic DNA per plate. When the recombinant clone was used, three different doses of 0.1, 1, and 10 ng per plate were used with NIH 3T3 DNA being added to make the 40-μg total. All the experiments were performed at least twice. The digestion with restriction enzymes was carried out in the conditions described by the manufacturers. The DNA's were subsequently extracted once with phenol and once with a mixture of chloroform and isoamyl alcohol (24:1 by volume) and precipitated with ethanol prior to transformation. When the donor DNA was λ3.2 N-*ras*T, the following enzymes, which cut inside the Kpn I–Xho I fragment, inactivated the gene: Hind III, Eco RI, Bam HI, Bgl II, Xba I, Pvu II, and Bal I. The transforming activity of cloned DNA treated with each of these enzymes was less than 12 foci per microgram of DNA. Conversely, with the same λ3.2 N-*ras*T donor DNA, the enzymes Sma I, Sst II, Sal I, Xho I, Kpn I, Pvu I, and Nru I yielded DNA that was still able to induce focus formation at a rate of 1×10^4 to 2×10^4 per microgram of DNA. These enzymes do not digest the insert.

Donor DNA	Transforming activity (foci/μg DNA)
Carcinogen-induced thymoma I	0.05 to 0.15
3T3-TI primary transformant	0.25 to 0.45
Rat 2-TI secondary transformant	0.30 to 0.45
λ3.2 N-*ras*T	1×10^4 to 2×10^4

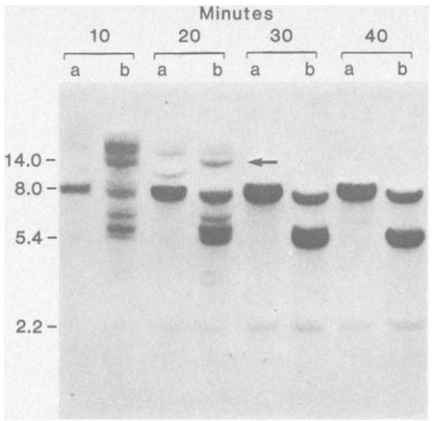
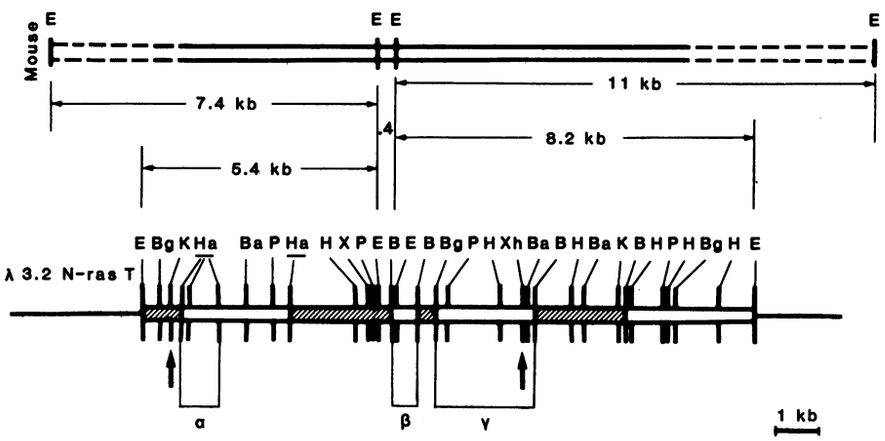


Fig. 1. Kinetics of digestion for rat and rat transformant DNA with Eco RI. Southern blot analysis of (a) rat DNA showing only the endogenous band at 8 kb and (b) rat transformant DNA showing the endogenous band at 8 kb and the transferred at 5.4 kb. The digestion was with Eco RI at increasing periods of time (10, 20, 30, and 40 minutes when the digestion was complete). Samples of DNA (200 μg) from rat and a rat transformant derived from an NMU-induced thymoma (tumor 1) were digested with 80 units of Eco RI under the standard conditions. The time chosen as ideal to proceed with the cloning was 20 minutes. At the times indicated, 20-μg portions were removed from each sample of DNA, EDTA was added to 20 m*M*, and the samples were chilled on ice until the last time point. They were then loaded in an 0.8 percent agarose gel, run at 70 V for 12 hours, and blotted by the Southern technique (*5*). Blotting, hybridization, and washing was as described (*4*). The hybridization was performed with a human N-*ras* specific probe [fragment R (*8*)]. The molecular weights were determined by comparison with comigrating λHind III fragments. The arrow signals the position of a 14-kb band present in the digest of the transformant and absent from the rat DNA and therefore is a partial product coming from the transferred mouse band. The numbers on the left are in kilobases. The rat DNA for the lanes labeled a was from a line in which the endogenous N-*ras* gene is amplified to give more sensitivity to the analysis.

jected to Southern blotting (*5*) (Fig. 1), a 14-kb DNA fragment homologous to an N-*ras* probe could be visualized in the transformed cell DNA but not in rat DNA. Because the transformed cells contain several copies of the mouse N-*ras* gene, this band was abundant even in partially digested DNA.

To clone this fragment, we fractionated the partially digested DNA by agarose gel electrophoresis (Fig. 1). DNA was eluted from the 10- to 15-kb region of the gel, checked by Southern blotting to confirm the presence of the 14-kb mouse fragment and absence of rat-specific fragments, and ligated into Eco RI digested λ47.1 vector DNA (*6*). A recombinant clone, λ3.2 N-*ras*T, containing an insert homologous to a human N-*ras* probe, was identified (*7*). This recombinant clone induced foci with an efficiency five orders of magnitude higher than that of total cellular DNA, demonstrating that a fully active N-*ras* oncogene had been cloned (Table 1).

We then constructed a restriction map of the recombinant clone (Fig. 2). Since we used a partial Eco RI digestion in the cloning process, we studied first the Eco RI map of the recombinant. The insert has three Eco RI fragments of 5.4, 0.4, and 8.2 kb. We used the 0.4-kb Eco RI fragment located in the middle of the cloned gene (Fig. 2) to probe Southern blots of Eco RI–digested rat and mouse DNA. The 0.4-kb fragment is only present in mouse DNA, as expected (data not shown). We also cloned the N-*ras* gene from mouse brain, and obtained an identical restriction map for the internal fragments, confirming that the cloned gene is of mouse origin.



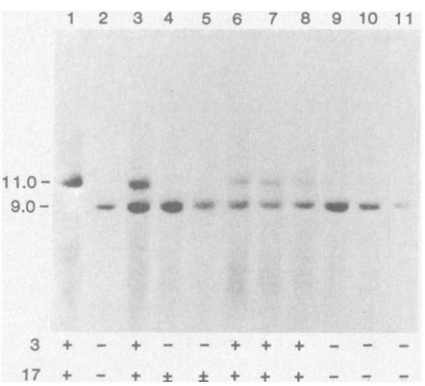


Fig. 2 (left). Restriction map of the clone λ3.2 N-*ras*T which contains a complete activated mouse N-*ras* oncogene. The map was constructed from a combination of single and double restriction enzyme digests of a λ3.2 N-*ras*T clone [^{32}P-labeled by nick translation (*18*)] and of subfragments of it. Digests were run in agarose gels, which were dried and autoradiographed. The regions containing mouse repetitive sequences were obtained from digests of λ3.2 N-*ras*T run in an agarose gel, Southern blotted, and hybridized with total mouse DNA ^{32}P-labeled by nick translation. The recombinant clone λ3.2 N-*ras*T is in λ47.1; phage sequences are the solid lines at both ends beyond the last Eco RI sites. The bar in between is the 14-kb insert. The hatched areas represent portions containing mouse repetitive sequences. The regions α, β, and γ indicate, respectively, the area where the human specific N-*ras* probe [fragment R (*8*)] hybridizes, a region containing one of the exons, identified with a specific probe from a human N-*ras* complementary DNA (*9*), and a region free of repetitive sequences which does not contain any coding exon but is at least in part essential for the transforming potential of the gene. The β region has been used to assign the gene to a chromosome (see Fig. 3). The upper drawing represents the information obtained with respect to the Eco RI fragments seen in mouse DNA. The length of the fragments encompassing regions with discontinuous lines has been inferred from Southern blots. The code for the enzymes is: E, Eco RI; Bg, Bgl II; K, Kpn I; Ha, Hae II; Ba, Bal I; P, Pvu II; H, Hind III; X, Xba I; B, Bam HI; Xh, Xho I. The underlined symbols are not mapped through all the insert. The arrows below the map indicate the smaller piece that still retains full transforming ability. Fig. 3 (right). Mouse N-*ras* gene in mouse × Chinese hamster somatic cell hybrids. Eco RI–digested genomic DNA was analyzed by Southern blotting (*19*) with fragment β (Fig. 2) being used as a probe. Sizes of genomic DNA fragments in kilobases are shown. Genomic DNA samples were from the BALB/c (mouse) cell line Meth A (lane 1), the Chinese hamster cell line E36 (lane 2), and from mouse × E36 hybrid cell lines BEM1-4 (lane 3), MACH 4A63 (lane 4), MACH 4B31AZ3 (lane 5), MACH 2A2B1 (lane 6), MACH 2A2C2 (lane 7), MACH 2A2H3 (lane 8), MAE 28 (lane 9), MAE 32 (lane 10), and ECm4e (lane 11). The propagation and karyotypic analysis of these cell lines have been described (*13*). The mouse parent of the BEM1-4 cell line was a BALB/c embryonic fibroblast; MACH hybrids were derived from A/HeJ macrophages; MAE hybrids from Meth A (BALB/c) cells, and ECm4e from L-cell sublines (C3H). Mouse chromosomes were detected in the somatic cell hybrids by karyotypic analysis as described previously (*13*), and the presence or absence of chromosomes 3 and 17 are indicated: −, <0.15 copies per cell; ±, 0.15 to 0.35 copies per cell; +, >0.35 copies per cell.

Using a combination of restriction digestion and transformation of NIH 3T3 cells, we identified an 8-kb Kpn I–Xho I fragment (Fig. 2) that is able to transform with the same efficiency as the whole clone. We then located on the restriction map the area homologous to the human probe used to identify the N-*ras* sequences in Southern blots of rodent DNA and to isolate the clone λ3.2 N-*ras*T. This sequence [fragment R from Shimizu *et al.* (*8*)] is specific for N-*ras* and corresponds to α in Fig. 2.

These data together provided enough information to approximately locate the ends of the gene. One end had to be located between the Kpn I site on the left of the map and the Bal I site nearby, because Kpn I does not destroy the transforming activity and Bal I does, and there is only one Bal I site in the 8-kb Kpn I–Xho I fragment. Since we know that in humans the fragment R, located close to the 5′ end, is part of the N-*ras* gene (*8, 9*) and its mouse counterpart α hybridizes with the N-*ras* transcripts (*10*), we could state that the 5′ end of the mouse gene should not be more than 1 kb inside the Kpn I site. Parenthetically, since Bgl II inactivates the gene and there is only one Bgl II site in the Kpn I–Xho I fragment, the 3′ end must be between this Bgl II site and the Xho I site. The size of the gene will be therefore a minimum of 6 and maximum of 8 kb. Our results indicate that the mouse N-*ras* gene (<8 kb) is approximately the same size as its human counterpart (*9, 11*) and the intronic and exonic structures are similar (*10*). Regions of the clone that lack mouse repetitive sequences (Fig. 2) were identified by hybridizing digests of the recombinant phage with radioactively labeled total mouse DNA.

We used the fragments α and β as probes to characterize the mouse genomic N-*ras* gene further. All inbred mouse strains examined (Figs. 2 and 3 and data not shown) contain a single DNA fragment of approximately 7.4 kb reactive with the α probe and one of 11 kb reactive with the β probe. The Eco RI sites that form the 5′ and 3′ ends of the insert in λ3.2 N-*ras*T appear therefore to have been generated by a rearrangement of sequences flanking the N-*ras* gene in the course of DNA-mediated gene transfer (*12*).

To determine the normal chromosomal location of N-*ras*, we tested a panel of somatic cell hybrids containing various combinations of mouse chromosomes on a constant Chinese hamster background for the presence or absence of the single 11-kb mouse-specific N-*ras* fragment (*13*). Preliminary results indicated that the genomic N-*ras* fragment was unlinked to previously mapped H- and K-*ras* loci. Ryan *et al.* (*14*) have mapped the native N-*ras* protooncogene to mouse chromosome 3. Analysis of a more extensive panel of somatic cell hybrids with the N-*ras* β probe yields a result consistent with this assignment (Fig. 3). There is no evidence of N-*ras* amplification or rearrangement in the thymus (*4*), further supporting the conclusion that the location of the activated gene is the same as the normal one.

It will now be interesting to analyze the mechanisms of N-*ras* activation in other thymic tumors induced by carcinogens and to determine whether the system described here is similar to the point mutation described in humans (*15*) and in other models (*4, 16*). Since bone marrow may contain the precursor cells for these thymic tumors, it may be possible to introduce this activated oncogene into bone marrow cells by transfection or through a retroviral vector and demonstrate involvement of N-*ras* in oncogenesis.

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Cadmium-Resistant *Pseudomonas putida* Synthesizes Novel Cadmium Proteins

Abstract. *Three cysteine-rich proteins of molecular weight 4000 to 7000, containing 4 to 7 gram atoms of cadmium, zinc, and copper per mole were isolated from* Pseudomonas putida *growing in 3 m*M *cadmium. The three proteins were induced during different phases of growth, and the smallest (molecular weight 3600; 3 gram atoms of cadmium) was released into the medium when the cells lysed. The results of amino acid analyses and of ultraviolet, circular dichroism, electron paramagnetic resonance, and cadmium-113 nuclear magnetic resonance spectroscopy suggest a novel cadmium(II)–zinc(II)–copper(I) cluster structure for the major protein.*

In many bacteria, cadmium resistance involves exclusion of cadmium (*1, 2*); for *Staphylococcus aureus*, this is achieved with an energy-dependent efflux system (*3, 4*). In contrast, *Pseudomonas putida* actively accumulates cadmium from the medium, and the resistance mechanism involves both polyphosphate and a series of low molecular weight cysteine-rich cadmium proteins that are induced during different growth phases. We describe the isolation and characterization of these proteins. In particular, a ^{113}Cd nuclear magnetic resonance (NMR) study on the major, native cadmium protein ($CdBP_1$) establishes a definite relationship to cadmium metallothioneins. Metallothioneins have been isolated from diverse organisms, including mammals (*5, 6*), yeast (*7*), algae (*8*), and fungi