Molecular cloning and chromosome assignment of murine *N-ras*

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ABSTRACT

The murine *N-ras* gene was cloned by screening an EMBL-3 recombinant phage library with a human *N-ras* specific probe. Hybridization of two separate unique sequence *N-ras* probes, isolated from the 5' and 3' flanking sequences of the murine gene, to a mouse-Chinese hamster hybrid mapping panel assigns the *N-ras* locus to mouse chromosome three.

INTRODUCTION

DNA-mediated gene transfer of DNA from transformed cells into NIH 3T3 cells has frequently identified members of the *ras* family of oncogenes as the transforming sequences (1,2). The transforming genes of the T-24 bladder carcinoma and Calu-1 lung carcinoma cell lines have been identified as activated *c-H-ras* and *c-K-ras*, respectively (3-6). These genes are closely related to the transforming genes of the Harvey and Kirsten murine sarcoma viruses. A third member of the *ras* family, *N-ras*, has been found to be activated in a neuroblastoma cell line, SK-N-SH (7,8). This gene has no known viral counterpart. The introduction by gene transfer into primary mouse cells of both an activated *ras* oncogene and an activated *myc* oncogene results in transformation, suggesting *ras* activation is one step in a multistep process of transformation (9,10). Activated *c-K-ras* has been found in various types of primary human tumor isolates, including lung carcinoma and ovarian carcinoma; in these cases the activated oncogene was found specifically in the tumor tissue and not in the patient's normal tissues (11-13). Activated *N-ras* has been seen in primary tumor isolates from colon and acute myelogenous leukemia tumors, and in bone marrow cells of a patient with acute myeloblastic leukemia (2,14,15). Activated *c-K-ras* and *N-ras* have also been found in chemically and radiation induced tumors in mice and in chemically induced benign skin papillomas (16-20).

In those cases where the activation mechanism is known the activated

ras oncogenes have point mutations introducing single amino acid substitutions in either of two regions of the ras p21 protein (21-26). Evidence for activation of c-K-ras in a mouse adrenocortical tumor by amplification has also been reported (27).

The ras oncogenes have been mapped in humans by somatic cell hybrid panel mapping and by in situ hybridization (for review, see 28). Human N-ras has been mapped to 1p11-13 (29-32). In the mouse c-H-ras has been assigned to chromosome seven (33,34) and c-K-ras to chromosome six (35). There is no obvious connection between the locations of the ras oncogenes in humans and mice and chromosome changes associated with tumors of various types (36).

To facilitate further studies of the role of N-ras in tumorigenesis using a mouse model, we have cloned the mouse N-ras gene from an EMBL-3 phage library. Here we present a preliminary characterization of the gene and an assignment of the genomic locus to mouse chromosome three.

MATERIALS AND METHODS

Molecular Probes

pNP-5 contains a 1.8 kb Xba I/Eco RI fragment of the 3' flanking region of the transforming N-ras oncogene of the SK-N-SH neuroblastoma cell line, cloned in pchk-2 (a pBr 322 derivative containing the chicken thymidine kinase gene) (7,26).

pNP-1 contains a 1.0 kb Hind III fragment, including the first exon, of the SK-N-SH N-ras oncogene (7,26).

N8.5 is an 8.5 kb Xba I/Sal I fragment isolated from recombinant phage 95a-4 and containing sequences homologous to the 5' flanking region of the murine N-ras gene.

N2.7 is a 2.7 kb Hind III/Sal I fragment isolated from recombinant phage 15a-2-4 and containing sequences homologous to the 3' flanking sequences of the murine N-ras gene.

N3.8 is a 3.8 kb Hind III/Sal I fragment isolated from recombinant phage 17b-4 and containing sequences homologous to the 3' flanking region of the murine N-ras gene.

N8.5, N2.7 and N3.8 were cut from 1% agarose gels and electroeluted. They were purified by running through an elutip-d column (Schleicher and Schuell) or by phenol/chloroform extraction. pNP-1 and pNP-5 were kindly provided by Dr. Michael Wigler.

Genomic Library

A lambda phage genomic library was constructed with the replacement

vector EMBL-3 (37). Mouse (CD-1) target DNA was prepared by partial digestion with Sau IIIa. 15-20 kb fragments were isolated from 0.8% agarose gels and purified by phenol and chloroform extraction. The phage vector DNA was prepared by subsequent Eco RI and Bam HI digests yielding Bam HI ends on the phage arms for cloning and Eco RI ends on the replaceable internal fragment. Vector and target DNA were ligated and then in vitro packaged according to the procedure of Ish-Horowitz (38). 2×10^6 viable recombinants were used to infect the K802 strain of E. coli and plated at a density yielding 2×10^4 plaques per 150-mm plate. The resulting plaques were transferred to nitrocellulose filters according to the procedure of Benton and Davis (39). Filters were washed, pre-hybridized, and hybridized according to procedures outlined in Maniatis, et. al. (40). The hybridization probe (pNP-1; corresponding to the 5' end of the human N-ras locus) was nick translated in the presence of ^{32}P dCTP to a specific activity of 1×10^8 cpm/ug. Filters were hybridized for 12 hours with 5 ng of probe per ml of hybridization mix and washed with 2x SSC/0.1% SDS and 0.2x SSC/0.1% SDS at 56°C . Kodak XAR-5 X-ray film was exposed to the filters for three days with an intensifying screen (Dupont) at -70°C . Positive plaques were plaque purified and a 500 ml batch of phage particles was prepared through a two-step amplification. Phage DNA was purified by the formamide method of Davis, et. al. (41).

Cell Lines

Mouse-Chinese hamster hybrids were constructed and characterized (by karyotype and isozyme analysis) as previously described (33). In addition, hybrids of the IF-4 series were analysed using a mouse chromosome three specific probe (pTSH- β , containing a cDNA coding for the β subunit of thyrotropin) kindly provided by Dr. James Gurr.

Southern Blotting and DNA Hybridization

DNA samples (0.25 ug of phage DNA or 25 ug of genomic DNA) were digested with restriction endonuclease (NEB or BRL, according to manufacturer's specifications) and electrophoresed in 1% agarose gels prior to Southern transfer (42) to nitrocellulose (Schleicher and Schuell). Hybridization was done at $60-65^\circ\text{C}$, 6x SSC, 2x Denhardt's, 100 ug/ml denatured, sonicated salmon sperm DNA for twelve hours with 10 ng/ml of denatured, nick translated probe added; 10% dextran sulphate (Pharmacia) was added for hybridizations to genomic DNAs. Final wash was either 65°C , 2x SSC, 0.01% SDS (low stringency) or 65°C , 0.1x SSC, 0.01% SDS (high stringency).

Figure 1. Restriction map of the mouse genomic region encompassed by phage inserts 17b-4, 92b-4, 15a-2-4, and 95a-4. Restriction fragments containing homologous sequences to the human *N-ras* probes, pNP-5 and pNP-1, are indicated. Sites outside of the four phage, mapped by genomic blots, are also indicated. The three unique sequence probes used in the mapping study (N3.8, N2.7 and N8.5) are identified alongside the phage they were isolated from. In phage 15a-2-4 and 95a-4 restriction fragments containing repetitive sequences are labelled with a black bar. Phage 17b-4 and 92b-4 gave identical results. The *Xba* I site labelled X* is present in 92b-4 and 15a-2-4, but not in 17b-4. Other sites: R- *Eco* RI, B- *Bam* HI, X- *Xba* I, H- *Hind* III, Bg- *Bgl* II, S- *Sst* I, K- *Kpn* I. The map of 95a-4 is not complete for *Hind* III and *Bgl* II. *Bgl* II, *Sst* I and *Kpn* I sites were mapped only in 95a-4.

RESULTS

Cloning and Characterization of Murine *N-ras*

pNP-1 was used as a probe to screen a CD-1 mouse genomic library constructed using lambda phage EMBL-3 as a vector. Four positive overlapping clones, 95a-4, 92b-4, 15a-2-4 and 17b-4, were isolated. Figure 1 summarizes the characterization of the cloned mouse sequences. Restriction fragments containing homologous sequences to the human *N-ras* probes, pNP-5 and pNP-1, are indicated; these are separated by approximately 5.5 kb of mouse DNA. Figure 2 shows hybridization of pNP-1 to the four recombinant phage, digested with three different restriction enzymes. In figure 3 the localization of pNP-5 to a homologous *Hind* III/*Bam* HI 1.9 kb mouse fragment of phage 15a-2-4 is shown. Similar results were obtained with phage 92b-4 and 17b-4. pNP-5 did not hybridize to phage 95a-4. Hybridization of the cloned DNA to total mouse genomic DNA allowed identification of restriction fragments containing repetitive elements (data not shown). Three unique sequence probes were isolated, a 3.8 kb *Sal* I/*Hind* III fragment from phage 17b-4, a 2.7 kb *Sal* I/*Hind* III fragment from phage 15a-2-4, and an 8.5 kb *Sal* I/*Xba* I fragment from

Figure 2. Human N-ras probe pNP-1 hybridized to recombinant phage. Tracks A-D: 15a-2-4, 95a-4, 92b-4, 17b-4, Hind III digest. Tracks E-H: 15a-2-4, 95a-4, 92b-4, 17b-4, Eco RI digest. Tracks I-L: 15a-2-4, 95a-4, 92b-4, 17b-4, Sal I digest. Sal I digestion separates the mouse insert from the EMBL-3 vector.

95a-4. Figure 4 shows probe N2.7 hybridized to mouse and Chinese hamster genomic DNA. This allowed localization of three genomic restriction sites lying 3' to the cloned mouse sequences (figure 1). N3.8 and N8.5 also gave hybridization patterns with mouse genomic DNA consistent with the restriction map shown in figure 1 (data not shown). Under high stringency conditions N2.7 does not cross-hybridize with hamster genomic DNA (figure 4). This is true also of probes N3.8 and N8.5 (data not shown).

Murine N-ras Maps to Chromosome 3

Figure 5 shows probe N2.7 hybridized to Hind III-digested DNA of six

Figure 3. Human N-ras probe pNP-5 hybridized to homologous mouse sequences. Track A: 3.2 kb Hind III fragment isolated from 15a-2-4. Track B: Bam HI digest of the 15a-2-4 3.2 kb Hind III fragment; hybridization is seen to the resulting 1.9 kb Bam HI/Hind III fragment.

Figure 4. Probe N2.7 hybridized to Mouse (A9) and Chinese Hamster (E36) genomic DNAs. Track A-F: A9 Hind III-cut, E36 Hind III-cut, A9 Bam HI-cut, E36 Bam HI-cut, A9 Eco RI-cut, E36 Eco RI-cut.

mouse-Chinese hamster hybrid cell lines. Positive tracks show a 7.0 kb band, as predicted from figure 1. Table 1 summarizes the results of hybridization of both N2.7 and N3.8 to forty hybrid cell lines; this data is consistent with a localization to mouse chromosome 3. Although one discordancy is seen for chromosome 3, there are at least five discordancies for every other chromosome, with the majority of chromosomes having greater than ten discordancies. The one discordancy observed for chromosome 3 could possibly be explained by a chromosomal rearrangement or translocation. Hybridization of probe N8.5 to a subset of the hybrid panel gave identical results (data not shown). Hybridization of pTSH- β , previously mapped to mouse chromosome 3 (43), to 15 hybrid DNAs of the IF-4 series gave an identical pattern as mouse N-ras, thus confirming the assignment to chromosome 3 (data not shown).

N-ras Related Sequences Map to Chromosome 14

Hybridization of N2.7 to mouse genomic DNA under low stringency conditions detects many (>15) cross-hybridizing bands (data not shown). Low stringency hybridization of N2.7 to Mae 29, a hybrid containing only mouse chromosomes 14 and X, detects one cross-hybridizing band (data not shown). Probe N8.5 also detects cross-hybridizing sequences, even at more stringent hybridization con-

Figure 5. Probe N2.7 hybridized to Hind III-digested Mouse-Chinese Hamster somatic cell hybrid DNAs. Track A: Meth(a) (Mouse control), Track B: E36 (Chinese Hamster control), Tracks C-H: TUCE 12G/4, MACH 4A63, 4B31 AZ3, mfe 2/1/2, cms 4, Mae 29. Hybrid mfe 2/1/2 was scored as positive.

ditions. Hybridization of N8.5 to Eco RI-digested mouse genomic DNA yields three bands (figure 6). A strongly hybridizing band of approximately 10 kb segregates with chromosome 3 and corresponds with the sequences contained in phage 95a-4 (see figure 1). There are also two lower molecular weight bands, one of 7.5 kb and one of 3.4 kb. As shown in figure 6, these smaller bands segregate independently of chromosome 3; the 7.5 kb band is concordant with

TABLE 1 Correlation of Blot Hybridization and Chromosome Status in Hybrids (N3.8 and N2.7 as probes)

presence of		Chromosome																			
hyb.	chrom.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	X
+	+	15	16	17	14	6	15	17	14	13	15	1	16	8	7	8	4	17	6	8	11
-	-	14	11	22	14	17	15	8	18	15	17	22	6	10	14	6	9	12	16	9	8
-	+	8	10	0	8	5	4	14	3	7	5	0	15	5	6	8	6	10	4	12	13
+	-	3	1	1	4	12	3	1	4	5	3	16	1	0	11	0	4	1	5	6	4
discordant		11	11	1	12	17	7	15	7	12	8	16	16	5	17	8	10	11	9	18	17

Figure 6. Probe N8.5 hybridized at low stringency to Eco RI-digested Mouse-Chinese Hamster somatic cell hybrid DNAs. Track A: A9 (Mouse control), Track B: E36 (Chinese Hamster control), Tracks C-J: IF-4 series of hybrids: C13, C11 ReHat II, C19 ReHat, C11 ReTG, C17A ReHat, Mc8 ReHat, Mcl, Mc8 TG. Mc8 ReHat was scored as positive for the 7.5 kb band.

mouse chromosome 14. Taken together with the N2.7 result, this suggests that sequences homologous to both the 5' and 3' flanking regions of N-ras are present on chromosome 14.

DISCUSSION

A comparison of the restriction map of the murine N-ras gene (figure 1) with published maps of the human N-ras gene (7,26) indicate that the murine gene is of approximately the same size. The sequences represented in pNP-1 and pNP-5 are separated by 8.2 kb of intervening DNA in the human gene (26). Hybridization of pNP-1 and pNP-5 to the cloned mouse N-ras sequences allowed localization of the homologous mouse sequences; as shown in figure 1, these sequences are approximately 5.5 kb apart in the murine gene. The intron-exon structure of the murine gene is yet to be determined, though the Xba I/Sst I fragment homologous to pNP-1 presumably contains the first exon of the murine gene. The region 5' to this fragment is characterized by a large segment of uninterrupted unique sequence DNA of at least 8.5 kb. The N8.5 probe isolated from this region cross-hybridizes to several related sequences in mouse genomic DNA (figure 6); at least one of the cross-hybridizing bands can be tentatively assigned to mouse chromosome 14. These results imply that a mouse N-ras multi-gene family exists, as has been previously demonstrated for human N-ras (7). A sequence which hybridizes to N2.7, a 3' flanking

region probe, at low stringency can also tentatively be assigned to chromosome 14, suggesting that a complete N-ras related gene resides on this chromosome. In contrast, mouse c-H-ras appears to have only a single homologous copy (44).

The assignment of the murine N-ras gene which is most homologous to human N-ras to mouse chromosome 3 is consistent with the previous assignment of human N-ras to chromosome 1p11-13 (29-32). Amylase 1 and amylase 2, which map to 1p221-q11 in man, map to chromosome 3 in the mouse (45). Human genes which map distal to amylase on the short arm of 1 have uniformly been mapped to mouse chromosome 4 (45). Those which map more distally on the long arm of 1 have been mapped to mouse chromosomes 1 or 4. In addition, human nerve growth factor gene maps to region 1p21 and the mouse nerve growth factor gene has been assigned to chromosome 3 (46,47). Thus, N-ras would appear to be part of an evolutionarily conserved linkage group including amylase 1, amylase 2 and nerve growth factor. The availability of cloned mouse N-ras probes will facilitate studies on N-ras activation in experimentally induced murine tumors.

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