Coordinate *N-ras* mRNA up-regulation with mutational activation in tumorigenic guinea pig cells

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ABSTRACT

Tumorigenic guinea pig cell lines with mutationally activated *N-ras* alleles also exhibited up-regulated *N-ras* mRNA. Mutational activation and mRNA up-regulation were limited to tumorigenic cells; preneoplastic progenitors were unaffected. Therefore, up-regulation occurred at a late stage of carcinogenesis closely associated with acquisition of tumorigenicity. cDNA and S1 protection analysis demonstrated that polyadenylation site of the short *N-ras* message and the mRNA start sites were different from that reported for human. The promoter region contained no canonical TATA or CCAAT boxes, but exhibited GGGCGG and CCGCCC SP1 binding motifs characteristic of growth control genes. Moreover, both mutant and wild-type alleles were up-regulated in a guinea pig line heterozygous for *N-ras* codon 61. Coordinate *N-ras* mutational activation and up-regulation in five independent tumorigenic lines with unique chromosome constitutions suggests that both events are required for expression of the neoplastic phenotype.

INTRODUCTION

Distinct, extended preneoplastic stages of guinea pig fetal cell transformation are advantageous for studying molecular carcinogenesis (1). Activation of a *ras* related gene during a late stage of carcinogenesis closely associated with acquisition of tumorigenicity occurred in five independent guinea pig cell lines initiated with diverse carcinogens (2, 3). Furthermore, all five lines contained an activated *N-ras* gene with the identical mutation, an AT to TA transversion at the third position of codon 61 which results in the insertion of histidine instead of glutamine (4). Moreover, each line, independent of carcinogen used, had a unique chromosomal constitution (5). The results suggested that the mutational event was not the direct result of the initiating carcinogen.

Sequence analysis of the guinea pig *N-ras* genomic clone demonstrated that the coding four exons as well as three non-coding exons observed in mature human *N-ras* mRNA were conserved (~95 and 80 to 90% homology to human *N-ras*, respectively; ref. 6). Moreover, heteroduplexes of human and guinea

pig genomic clones revealed at least 65% homology in extensive regions of *N-ras* introns (6). The high degree of conservation of non-coding regions suggests that they have a function in control of *N-ras* expression. Increased levels of *N-ras* mRNA have been observed in human tumor cells containing activated *N-ras* alleles (V. Notario, personal communication) and increased expression of an *N-ras* gene under the control of an inducible promoter transfected into NIH/3T3 cells resulted in corresponding increased states of transformation (7). Because changes in *N-ras* expression may be an important aspect of carcinogenesis, the regulation of *N-ras* mRNA in guinea pig transformed cells was examined.

MATERIALS AND METHODS

Cells and Culture.

Tumorigenic guinea pig cell lines, 74C3, 104C1, 107C3, HM2C1, and HM2C1, their preneoplastic progenitor cell lines, and normal guinea pig cells, GP84, have been described (1). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37 C in a 5% CO₂ atmosphere.

RNA Isolation and Analysis.

RNA extraction was performed according to the procedures of Ullrich *et al.* (8). Poly(A)-terminated RNA was prepared by the method of Aviv and Leder (9), electrophoresed in formaldehyde gels (10), and electroblotted onto GeneScreen. Single-stranded S1 DNA probes were prepared by the method of Ley *et al.* (11); antisense *N-ras* specific oligonucleotides (21-mers) annealed to M13 single-stranded DNA templates were extended *in vitro*. Preferential labelling of the 5'-end was achieved by pulse labeling with high specific activity [³²P]dATP, 3000 Ci/mmol (10 mol dATP/mol DNA template), and chasing with cold dATP. The S1 probes were purified by restriction at the M13 cloning site and alkaline agarose electrophoresis. S1 analysis was performed by the procedure of Berk and Sharp (12) with 5 µg poly(A)-terminated RNA and 5 X 10⁶ cpm of probe. The sizes of protected fragments were determined on DNA sequencing gels; marker lanes were generated from dideoxynucleotide sequencing reactions using the same oligonucleotide and M13 template utilized in constructing the S1 probe.

cDNA Cloning and Analysis.

cDNA was synthesized by the Gubler/Hoffman technique (13) with Moloney Murine Leukemia Virus Reverse Transcriptase using the reagents and protocols of the Bethesda Research Laboratory's cDNA synthesis kit. The cDNA was cloned into the *Eco* RI site of λ gt10 using Amersham's cDNA cloning system.

The library was screened with a human *N-ras* cDNA clone containing exons II - IV obtained from Oncogene Science Inc. For sequence analysis by the Sanger method (14) cDNAs were recloned into M13mp19 (15); oligonucleotide primers (21-mers) were synthesized with sequences determined from the guinea pig genomic *N-ras* clone, 107C3/B5. Oligonucleotide hybridization was used as previously described to ascertain from which *N-ras* allele each cDNA was derived (4, 16).

RESULTS AND DISCUSSION

Up-regulation of *N-ras* mRNA in tumorigenic cells

To determine the importance of increased *N-ras* mRNA to guinea pig carcinogenesis, Northern blot analysis with mRNA from five independent tumorigenic lines and normal guinea pig cells was performed (Fig. 1A). All cell lines expressed the two major guinea pig *N-ras* mRNA species, 1.25 and 4.8 kb. Moreover, the level of *N-ras* messages was 10 to 20 fold higher

Figure 1. Up-regulation of *N-ras* mRNA in tumorigenic guinea pig cells. 5 µg of poly(A)-terminated RNA from each cell line was electrophoresed in formaldehyde gels and transferred to GeneScreen (New England Nuclear). The poly(A)-terminated RNA fraction eluted from oligo(dT) cellulose columns was 4 to 5% of the total RNA in all cases. The Bethesda Research Laboratories' RNA ladder were used as markers. A) Comparison of tumorigenic and normal (GP84) cells. The filter was probed with the *Pst* I fragment of guinea pig genomic clone 107C3/B5 containing *N-ras* exon II (4). B) Control comparison. The filter in A) was stripped and probed with a GR FeSV derived γ -actin specific DNA fragment (17) to quantify the amount of mRNA in each lane. C) Comparison of preneoplastic ('P' designates cell passage) and normal cells. Blots A and C should not be compared quantitatively because different probe preparations and exposure times were used.

Figure 2. Relationship between guinea pig *N-ras* genomic and cDNA clones. The exons on clone 107C3/B5 (4) are indicated by solid bars and roman numerals. Clone 107C3/c9 is also representative of clones 107C3/c3 and c8. The sequence in the region of the polyadenylation site of the short guinea pig message was taken from Doniger (6); letters above the sequence indicate differences in the human gene (18); the dash in the sequence is a deletion inserted to maximize homology. The polyadenylation signal and site are underlined. 3'-ends of cDNA clones are indicated below the sequence. B, *Bam* HI; E, *Eco* RI; P, *Pst* I; S, *Sst* I; A_n, poly(A) tail.

(determined by densitometer analysis) in all the transformed lines compared to normal ones. Furthermore, a 2 kb band was detected with the same intensity in all the lanes with a γ -actin probe (17) used on the same blot, demonstrating that the same amount of mRNA from normal and transformed cells was analyzed (Fig. 1B). (The additional 1.7 kb species observed in GP84 mRNA is presumably the transcript of a fetal γ -actin related protein that was down-regulated after extensive culturing). Therefore, *N-ras* up-regulation, i. e. an increased level of mature mRNA, is an integral aspect of guinea pig cell transformation.

Mutational activation of *N-ras* was observed only in tumorigenic cells (2). *N-ras* mRNA levels in preneoplastic progenitor cells of three of the tumorigenic guinea pig lines were measured to determine whether *N-ras* up-regulation preceded its activation (Fig. 1C). Again, the two major *N-ras* mRNA species were found, but the level of *N-ras* messages in all of the preneoplastic cells was similar to that in normal guinea pig cells. Thus, *N-ras* up-regulation occurred at a late stage of carcinogenesis, closely

associated with acquisition of tumorigenicity and *N-ras* mutational activation.

N-ras mRNA structure

cDNA clones were isolated from cell line 107C3 to gain further insight into the control of *N-ras* expression. cDNA was cloned into the *Eco* RI site of λ gt10; the library was screened with a human *N-ras* cDNA probe. From 8×10^4 clones screened ten *N-ras* were found; all were between 0.9 to 1.6 kbp. Clones 107C3/c2, c3, c8, and c9 were studied extensively by DNA sequence analysis. All contained seven genomic segments (Fig. 2) with the same intron/exon junctions as described for a human cDNA (18) and predicted from the guinea pig genomic sequence (6). However, both the 3'-end of the short *N-ras* message and the 5'-end differed from that reported for the human.

The 3'-end Clones 107C3/c3, c8, and c9 were all ~1.2 kbp, similar to the size of the small *N-ras* message. Clones 107C3/c8 and c9 end 397 and 420 bases, respectively, downstream from the beginning of exon VI but have no poly(A) tail, whereas clone 107C3/c3 has a poly(A) tail after the 420th or 421st base of exon VI; the 421st base in the genomic sequence is an A (Fig. 2). The sequence CATAAA, close to the consensus polyadenylation signal, AATAAA (19), at base 396 must be the polyadenylation signal. Furthermore, the triple AATAAA sequence, about 230 bases downstream, previously considered on the basis of sequence analysis to be the polyadenylation signal for the short guinea pig message (6), does not function. In contrast, the polyadenylation site of the short human message is at the 1087th base of exon VI (18). Moreover, the CATAAA sequence is conserved in human exon VI (Fig. 2); only three base differences were found between the polyadenylation signal and site. Another 12 differences occur in the next 50 bases downstream where polyadenylation enhancer signals (20) usually exist. No obvious polyadenylation enhancer sequences were recognized in this region. Presumably, one or several of these differences are responsible for the failure of human *N-ras* polyadenylation at this site.

Clone 107C3/c2 (1.6 kb) ends at the *Eco* RI site 398 bases downstream of the short message polyadenylation site. This clone was presumably derived from a cDNA of the long *N-ras* message which was *Eco* RI restricted during linker digestion before ligation to λ gt10 vector arms during cloning. This is consistent with the observation that the long human *N-ras* message contains an extended exon VI due to incomplete processing at the short message polyadenylation site (18). Moreover, the ratio of the short to long mRNA was 1.5 to 2 in guinea pig cells whereas it was 0.33 in humans.

Figure 4. *unr* is adjacent to the 5'-end of *N-ras*. 5 µg of poly(A)-terminated RNA from 107C3 cells was electrophoresed in formaldehyde gels and transferred to GeneScreen (New England Nuclear). The Northern blot was probed and stripped successively. Lane a, human *N-ras* cDNA with exons II to IV (Oncogene Sciences Inc.); lane b, clone 107C3/B5 (see Fig. 2); lane c, 9.5 kbp *Pst* I/*Bam* HI subfragment of clone 107C3/B5 with *N-ras* exons III to VI; lane d, 1.4 kbp *Pst* I subfragment of clone 107C3/B5 with exons -I and I; lane e, 2.8 kbp *Bam* HI/*Pst* I subfragment of clone 107C3/B5 (upstream of *N-ras* exons).

Therefore, the polyadenylation signal for the short message is relatively more effective in the guinea pig.

The 5'-end The 5'-ends of all four clones were within 25 bp of each other; clone 107C3/c9 had the largest exon -I, 101 bp. To determine the 5' end of the message more precisely, S1 analysis was performed (Fig. 3). The S1 probe, preferentially labeled at the 5'-end, was prepared by extension of an oligonucleotide primer that begins three bases upstream of the exon -I splice donor site. Most of the label was in the next 20 bases because 11 out of the first 20 bases in the template strand upstream of the primer are T and a 10:1 molar ratio of 32 P-labelled ATP to template DNA was used. Three major protected fragments were observed that coincide with positions

Figure 5. The sequence of *N-ras* codon 61 in the cDNA clones. cDNA clones were analyzed by oligonucleotide hybridization. DNA from λ 107C3/c clones was digested with *Eco* RI, electrophoresed in 0.7% agarose, and electroblotted onto GeneScreen. The filters were hybridized with 32 P-end labelled 19-mer oligonucleotides, 61CAA (wild-type; top row) or 61CAT (mutant; bottom row), as described (4). 62 C was the discriminating washing temperature.

in the sequence that are 113, 114, and 119 bases upstream from the exon -I splice donor site. These positions are 12 to 18 bases upstream from the 5'-end of clone 107C3/c9. Interestingly, CCAAT and TATA sequences are found 91 and 56 bp, respectively, upstream of exon I in the intron of the genomic guinea pig sequence. S1 protection analysis with an exon I probe demonstrated that this putative promoter site did not function (Fig. 3); no protected fragments that extended beyond the exon I splice acceptor site were observed. In summary, the total length of *N-ras* sequences in the short message deduced from cDNA and S1 analysis of 107C3 mRNA was 1170 bases, consistent with 1.25 kb determined by Northern blot analysis.

The size of human exon -I was purported as 225 -235 bp (18). Human cDNA

clone pcN1 contains only 48 bp upstream of the exon -I splice donor site. Therefore, S1 analysis had also been used to define the beginning of the human *N-ras* message. The probe used had been uniformly labeled and extended about 700 bases upstream of the exon -I splice donor site to a *Pst* I site. However, this probe could have also recognized a different gene. A gene, designated *unr* (upstream of *N-ras*), has been detected adjacent to *N-ras* by Northern blot analysis of guinea pig mRNA. Three *unr* transcripts, 3.0, 3.7, and 4.1, were observed in guinea pig mRNA probed with either the complete 107C3/B5 clone (Fig. 4, lane b) or the 2.8 kbp *Bam* HI/*Pst* I subfragment from its 5'-end (Fig. 4, lane e). These three transcripts are not *N-ras* messages because they were not detected when the same Northern blot was probed with human *N-ras* cDNA (lane a) or the 9.5 kbp *Pst* I/*Bam* HI subfragment of clone 107C3/B5 containing *N-ras* exons III through VI (lane c). When the 1.4 kbp *Pst* I subfragment of clone 107C3/B5 containing exons -I and I was used as a probe the predominant species was the *unr* 4.1 kb transcript (lane d). This probe was similar to the probe used in S1 protection analysis of the human *N-ras* promoter except that it also contained *N-ras* exon I sequences. Because greater than 80% homology between guinea pig and human DNA was observed in the region of *unr* by heteroduplex analysis of genomic *N-ras* clones and greater than 90% was found between the *Pst* I site by sequence analysis (6) it is likely that *unr* exists and is transcribed in human cells. Therefore, it is conceivable that the major species, 225 - 235 bp, detected by the S1 analysis of the human *N-ras* promoter region were protected by *unr* mRNA rather than *N-ras*. Under these circumstances human and guinea pig *N-ras* messages could have the same 5'-end as defined here.

The region upstream of the guinea pig *N-ras* message is GC rich, approximately 70% (Fig. 3). No canonical TATA or CCAAT boxes (21) exist in this region. However, the SP1 binding sequence, GGGCGG (or its complement CCGCCC), exist twice upstream of and once at the mRNA start region (Fig.3). This sequence has been observed in the SV40 21 bp repeat (22), upstream of cellular housekeeping genes (23-25), and upstream of K- and H-*ras* (26, 27).

Both mutant and wild-type *N-ras* alleles in 107C3 cells are up-regulated

107C3 cells, used for making the cDNA library, are heterozygous for the codon 61 *N-ras* mutation (4). Therefore, the cDNA clones were analyzed with oligonucleotide probes to determine whether either of these alleles were preferentially expressed (Fig. 5). Of the ten *N-ras* clones five were derived from the wild-type allele and five from the mutant; oligonucleotide analysis of clones 107C3/c2, 3, 8, and 9 was subsequently confirmed by

Figure 6. Expression of *H-ras* and *unr* in transformed guinea pig cells. The Northern blot used in Fig 1A and B was stripped and probed with viral *H-ras* DNA (Oncor) in A) and with the 2.8 kbp *Bam* HI/*Pst* I subfragment of clone 107C3/B5 (specific for *unr*) in B).

sequence analysis. Therefore, neither allele was expressed preferentially; both were up-regulated. *N-ras* up-regulation was the result of either a regulatory mutation in both *N-ras* alleles or, more likely, a change in a regulatory modulator. To determine whether mutations occurred in the promoter region, the sequences in this region of genomic clones 107C3/B5 and 107C3/B30, isolated from transformed cells, and GP84/B6, isolated from normal cells, were compared. No sequence difference was observed in clone GP84/B6 relative to 107C3/B5 or 107C3/B30 in the 341 bp upstream of exon -I start site; at this position a rearrangement was observed in clone 107C3/B30 that occurred during transformation of NIH/3T3 cells (4).

Up-regulation of *N-ras* could be part of a general up-regulation of cellular housekeeping genes in transformed guinea pig cells. To determine if this were true, the Northern blot from Figure 1A was probed with viral *H-ras* (Fig. 6A). The major *H-ras* transcript was 5.2 kb; a minor 0.85 kb species was also observed after a longer exposure. A minimal up-regulation of *H-ras* message was found in mRNA from transformed cells; two- to five-fold by densitometer analysis. This was significantly less than the 10- to

20-fold up-regulation of *N-ras*. If a common modulator for up-regulation of housekeeping genes exists, *N-ras* was much more responsive. The same Northern blot was also probed with a *unr* specific probe to ascertain whether up-regulation of *unr* and *N-ras* was coordinated (Fig. 6B). Between two- and eight-fold up-regulation of *unr* was detected. Even though this was less than the up-regulation of *N-ras* it might suggest that *N-ras* and *unr* share a common cis-acting enhancer element.

N-ras up-regulation occurred in all the guinea pig cell lines that contain the codon 61 mutation. Another means for *ras* gene activation has been proposed i. e. truncation of exon -I (28). Could the up-regulated *N-ras* message in tumorigenic guinea pig cells be truncated? No apparent size difference in *N-ras* mRNA was observed in normal and transformed cells. Thus, any truncation of the up-regulated message must be very small. Furthermore, the DNA sequences of the promoter region upstream of exon -I are the same in *N-ras* genomic clones from normal and tumorigenic guinea pig cells. Thus, it is not obvious why these *N-ras* alleles would promote mRNAs with truncated 5'-ends. However, the consistency of the observation of *N-ras* mutation with up-regulation in neoplastic guinea pig cells suggests that both of these events were necessary for full expression of the tumorigenic phenotype.

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REFERENCES

1. Evans, C. H., and DiPaolo, J. A. (1975) *Cancer Res.* 35, 1035-1044.
2. Sukumar, S., Pulciani, S., Doniger, J., DiPaolo, J. A., Evans, C. H., Zbar, B., and Barbacid, M. (1984) *Science* 223, 1197-1199.
3. Doniger, J. (1985) In Barrett, J. C. and Tennant, R. W. (eds), *Carcinogenesis: A Comprehensive Survey*, Vol. 9: Mammalian Cell Transformation, Mechanisms of Carcinogenesis and Assays for Carcinogens, Raven Press, New York, pp. 51-55.
4. Doniger, J., Notario, V. N., and DiPaolo, J. A. (1987) *J. Biol. Chem.* 262, 3813-3819.
5. Popescu, N. C., Evans, C. H., and DiPaolo, J. A. (1976) *Cancer Res.* 36, 1404-1413.
6. Doniger, J. (1987) *Oncogene* In press.
7. McKay, I. A., Marshall, C. J., Cales, C., and Hall, A. (1986) *EMBO J.* 5, 2617-2621.
8. Ullrich, A., Shine, J., Chirgwin, J., Pictet, R., Tischler, E., Rutter, W. J., and Goodman, H. M. (1977) *Science* 196, 1313-1319.
9. Aviv, H., and Leder, P. (1972) *Proc. Natl. Acad. Sci., USA* 69, 1408-1412.
10. Lehrach, H., Diamond, D., Wozney, J., and Boedtker, H. (1977) *Biochemistry* 16, 4743-4751.
11. Ley, T. J., Anagnou, N. P., Pepe, G., and Nienhuis, A. W. (1982) *Proc.*

- Natl. Acad. Sci. USA 79, 4775-4779.
12. Berk, A. J., and Sharp, P. A. (1977) *Cell* 12, 721-732.
 13. Gubler, U., and Hoffman, B. J. (1983) *Gene* 25, 263-269.
 14. Sanger, F., and Coulson, A. R. (1978) *FEBS Lett.* 87, 107-110.
 15. Norrander, J., Kempe, T., and Messing, J. (1983) *Gene* 26, 101-106.
 16. Conner, B. J., Reyes, A. A., Morin, C., Itakura, K., Teplitz, R. L., and Wallace, R. B. (1983) *Proc. Natl. Acad. Sci., USA* 80, 278-282.
 17. Naharro, G., Robbins, K. C., and Reddy, E. P. (1984) *Science* 223, 63-66.
 18. Hall, A., and Brown, R. (1985) *Nucleic Acids Res.* 13, 5255-5268.
 19. Proudfoot, N. J., and Brownlee, G. G. (1976) *Nature* 263, 211-214.
 20. Hart, R. P., McDevitt, M. A., Ali, H., and Nevins, J. R. (1985) *Mol. Cell. Biol.* 5, 2975-2983.
 21. Breathnach, R., and Chambon, P. (1981) *Annu. Rev. Biochem.* 50, 349-383.
 22. Benoist, C., and Chambon, P. (1981) *Nature* 290, 304-310.
 23. Ishii, S., Xu, Y. H., Stratton, R. H., Roe, B. A., Merlino, G. T., and Pastan, I. (1985) *Proc. Natl. Acad. Sci. USA* 82, 4920-4924.
 24. Melton, D. W., Konecki, D. S., Brennand, J., and Caskey, C. T. (1984) *Proc. Natl. Acad. Sci. USA* 81, 2147-2151.
 25. Reynolds, G. A., Basu, S. K., Osborne, T. F., Chin, D. J., Gil, G., Brown, M. S., Goldstein, J. L., and Luskey, K. L. (1984) *Cell* 38, 275-285.
 26. Hoffman, E. K., Trusko, S. P., Freeman, N., and George, D. L. (1987) *Mol. Cell. Biol.* 7, 2592-2596.
 27. Ishii, S., Merlino, G. T., and Pastan, I. (1985) *Science* 230, 1378-1381.
 28. Cichutek, K., and Duesberg, P. H. (1986) *Proc. Natl. Acad. Sci. USA* 83, 2340-2344.