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Mechanism of activation of an N-*ras* gene in the human fibrosarcoma cell line HT1080

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A full length N-*ras* gene has been cloned from both the human fibrosarcoma cell line HT1080 and from normal human DNA. N-*ras* isolated from HT1080 will efficiently induce morphological transformation of NIH/3T3 cells in a transfection assay, whereas N-*ras* isolated from normal human DNA has no effect on NIH/3T3 cells. The coding regions of the normal N-*ras* gene have been sequenced and the predicted amino acid sequence of the N-*ras* product is very similar to that of the c-Ha-*ras*1 and c-Ki-*ras*2 products. By making chimeric molecules between the two cloned genes the activating alteration in the HT1080 N-*ras* gene has been localised to a single base change that results in an amino acid alteration at position 61 of the p21 N-*ras* product.
Key words: fibrosarcoma/homologous recombination/human oncogene/N-*ras*/transfection assay

Introduction

A wide variety of human tumours have been found to contain genes that are capable of transforming NIH/3T3 cells in a DNA transfection assay (Murray *et al.*, 1981; Perucho *et al.*, 1981; Lane *et al.*, 1982; Marshall *et al.*, 1982). In the majority of cases these transforming genes, or oncogenes, have been found to be activated members of the *ras* gene family. Four *ras* genes were originally identified in the human genome on the basis of their cross-hybridisation to the viral *ras* oncogenes (Chang *et al.*, 1982). Two of these genes, c-Ha-*ras* 1 and 2, are closely related to the viral Harvey *ras* oncogene and two, c-Ki-*ras* 1 and 2, are more closely related to the viral Kirsten *ras* oncogene. Both the viral *ras* genes have been found to encode p21 transforming proteins. The mechanism of action of these proteins is not understood though it is known that the *ras* proteins bind GTP (Shih *et al.*, 1980) and are located at the inner surface of the cytoplasmic membrane (Willingham *et al.*, 1983). Furthermore, the viral *ras* p21 products are capable of autophosphorylation in the presence of GTP (Shih *et al.*, 1980) with the inference that the proteins may have a GTP kinase activity.

Activated c-Ha-*ras*1 genes have been detected in the bladder carcinoma cell lines EJ and T24 by several groups (Santos *et al.*, 1982; Parada *et al.*, 1982; Der *et al.*, 1982). It was later shown that the gene isolated from these tumour cell lines differed from the gene present in normal cells by a single base change, presumably as the result of a somatic mutation (Reddy *et al.*, 1982; Tabin *et al.*, 1982; Taparowsky *et al.*, 1982). The consequence of this mutation, located in the first coding exon of the gene, is to alter the 12th amino acid from glycine to valine in the p21 transforming protein. Another example of an activated c-Ha-*ras*1 gene has been characterised from the lung carcinoma cell line HS242 (Yuasa *et al.*, 1983). In this case a single base change results in the substitution of leucine for glutamine at position 61 within the second exon.

A second activated member of the *ras* gene family, c-Ki-*ras*2, has been detected in a large number of different tumour cell lines (Pulciani *et al.*, 1982; Der *et al.*, 1982; McCoy *et al.*, 1983). The precise mechanism of activation has been determined for two cell lines, SW480, a colon carcinoma, and Calu-1, a lung carcinoma (Shimizu *et al.*, 1983; Capon *et al.*, 1983). In both cases there is a single base change at the codon for amino acid 12. In SW480, this results in the replacement of glycine by valine, while in Calu-1 glycine is replaced with cysteine. There have been no reported cases of c-Ha-*ras*2 or c-Ki-*ras*1 activation and it appears that both are functionless pseudogenes (McGrath *et al.*, 1983; Miyoshi *et al.*, 1984).

We and others have identified a fifth member of the *ras* gene family, N-*ras*, using the DNA transfection assay. We have shown that N-*ras* is a distinct member of the *ras* gene family and is located on the short arm of chromosome 1 just above the centromere (Hall *et al.*, 1983; Davis *et al.*, 1983). An activated form of this gene has been detected in a variety of cell lines, including those derived from sarcomas, neuroblastomas and leukemias (Hall *et al.*, 1983; Taparowsky *et al.*, 1983; Murray *et al.*, 1983; Eva *et al.*, 1983; Gambke *et al.*, 1984). The N-*ras* gene isolated from the neuroblastoma cell line, SK-N-SH, has been activated by a single base change resulting in the alteration of a glutamine residue at amino acid 61 to a lysine residue (Taparowsky *et al.*, 1983). We report here the cloning of a full length activated N-*ras* gene from the human fibrosarcoma cell line HT1080, the cloning of a normal N-*ras* gene from fetal liver DNA, and the determination of the mechanism of activation of the gene in HT1080.

Results

We have reported the isolation from HT1080 transformed NIH/3T3 cells of an 8.8-kb *Eco*RI fragment containing a portion of the activated N-*ras* gene (Hall *et al.*, 1983). We further showed that an overlapping 11-kb *Hind*III fragment contained the remainder of this gene. Consequently DNA from an HT1080 transfectant was digested with *Hind*III and a library constructed in the phage vector L47.1 (Loenen and Brammar, 1980). The library was screened with probe A (Figure 1a) and a clone was obtained which overlapped with the previously isolated 8.8-kb *Eco*RI fragment. A detailed restriction map of this region is shown in Figure 1a. It can be seen that the gene is cut by a single *Eco*RI site into two fragments, 8.8 kb and 7.0 kb. From a preliminary analysis of human DNA present in a number of NIH/3T3 transfectants we predicted that the active gene lay between the *Bam*HI site, located in the 8.8-kb *Eco*RI fragment and the *Sst*I site in the 7.0-kb *Eco*RI fragment (Hall *et al.*, 1983). To confirm this we

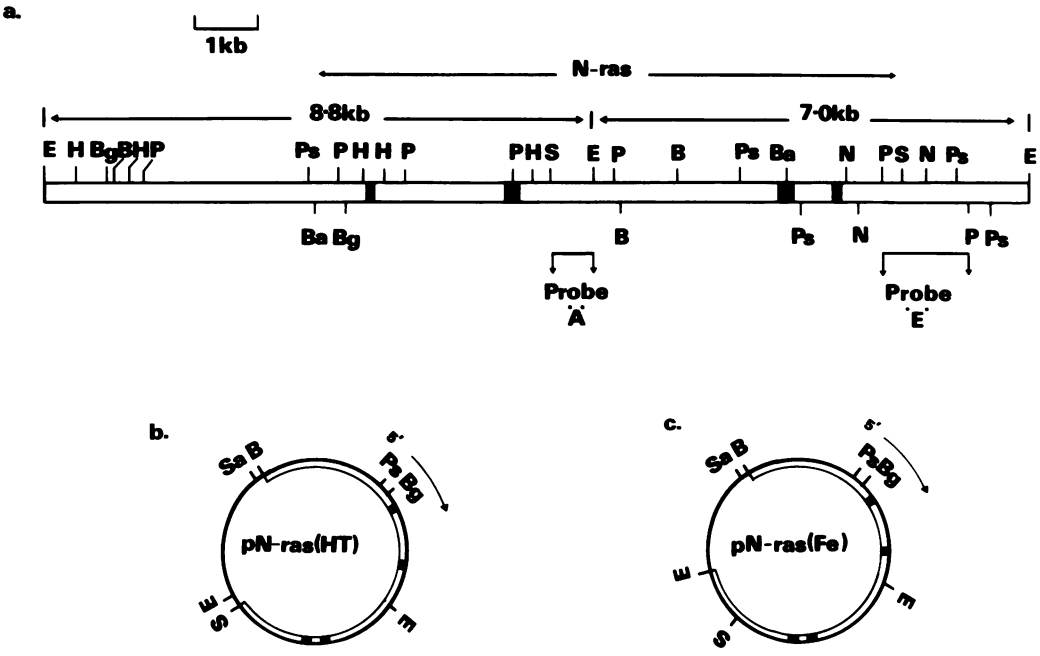

Fig. 1. The N-*ras* gene. **(a)** Restriction map of the N-*ras* gene isolated from HT1080. The gene is conveniently split into two by an *Eco*RI site yielding an 8.8-kb fragment from the left-hand end and a 7.0-kb fragment from the right end. The restriction sites are as follows: B, *Bam*HI; Ba, *Bal*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; N, *Nde*I; P, *Pvu*II; Ps, *Pst*I; S, *Sst*I; Sa, *Sal*I. The restriction map is complete for these enzymes except for *Pst*I, *Nde*I and *Bal*I in the 8.8-kb fragment and *Bal*I in the 7.0-kb fragment. The 7.0-kb *Eco*RI fragment was isolated as part of an 11-kb *Hind*III fragment. The left-hand end of this *Hind*III fragment is within the 8.8-kb *Eco*RI fragment and the right-hand end has not been included in the restriction map since it is not relevant. The four coding exons are shown as black boxes. The known boundaries of the gene (see text) are also indicated. **(b)** Plasmid pN-*ras* (HT) containing a biologically active gene. The single line *Sst*I to *Bam*HI represents vector sequences derived from pHLTR (Chang *et al.*, 1982). The approximate positions of the exons are shown within the N-*ras* sequence and the *Pst*I site at the 5′ end of the gene is also shown. **(c)** Plasmid pN-*ras* (Fe) containing a full length N-*ras* gene isolated from fetal liver DNA. The single line *Bam*HI to *Eco*RI represent vector sequences derived from pAT153.

constructed a plasmid, pN-*ras* (HT), containing this segment of DNA (see Figure 1b). This plasmid morphologically transformed NIH/3T3 cells with an efficiency of ~2000 foci/μg of plasmid DNA.

Boundaries of the N-ras gene

To map more closely the boundaries of the N-*ras* gene present in the clone, deletions were constructed and tested for biological activity. Since we have shown that the plasmid pN-*ras* (HT) has transforming activity then the 3′ end of the gene must be upstream of the *Sst*I site present in the 7.0-kb *Eco*RI fragment (Figure 1a). Furthermore, although probe E (Figure 1a) hybridises to N-*ras* mRNA on Northern blots it does not contain any coding sequence (see later Results). We predict, therefore, that the 3′ end of the 2.2-kb N-*ras* mRNA lies within a 300 bp stretch of DNA between the *Sst*I site and a *Pvu*II site located just upstream (Figure 1a). Interestingly probe E also hybridises to a 5.2-kb mRNA which is present in HT1080 and in some, though not all, transfectants. This species appears to contain an elongated 3′-untranslated sequence that is derived from genomic sequences extending through the *Eco*RI site at the end of the 7.0-kb fragment (unpublished results).

To map the 5′ end of the gene, a variety of fragments were isolated from the 8.8-kb *Eco*RI fragment and ligated to the large *Bgl*II-*Sal*I fragment from pN-*ras* (HT) (see Figure 1b) that contains all of the four coding exons plus the 3′ end of the gene. The DNAs were then tested in transfection assays. As expected, the *Bgl*II/*Sal*I fragment itself has no transforming activity (no foci from 1 μg of DNA), whereas ligation to a *Bam*HI/*Bgl*II fragment, effectively recreating pN-*ras* (HT), results in transforming activity (30 foci from 0.3 μg of DNA from the mixed ligation). Ligation of a *Pst*I/*Bgl*II fragment to the *Bgl*II/*Sal*I fragment similarly restores its biological activity (25 foci from 0.3 μg of DNA). We conclude from these results that the 5′ end of the N-*ras* gene lies 3′ to the *Pst*I site indicated in Figure 1.

Isolation of a normal N-ras gene

We made use of the fact that the N-*ras* gene contains only a single internal *Eco*RI site to isolate a full length normal N-*ras* gene from human fetal liver DNA. The DNA was partially digested with *Eco*RI and sedimented through a potassium acetate gradient. Fractions were collected and analysed by Southern blots using a probe for either the 8.8-kb *Eco*RI fragment (probe A Figure 1a) or for the 7.0-kb *Eco*RI fragment (probe E Figure 1a). A fraction was identified which contained DNA with an average size of 16 kb and which was enriched for the 8.8- and 7.0-kb sequences. This was then used to construct a library in the phage vector L47.1, and the recombinants were selected by growth on a P2 lysogen. The library was screened with probe A and a clone was identified which contained both *Eco*RI fragments, i.e., a full length N-*ras* gene. This clone had an identical restriction map to that obtained from HT1080. The normal N-*ras* gene, from the *Bam*HI site to the second *Eco*RI site, was subcloned into *Bam*HI/*Eco*RI sites of pAT153 to yield pN-*ras* (Fe) (see Figure 1c) and as expected this clone gave no transformed foci when assayed on NIH/3T3 cells even using up to 10 μg of plasmid DNA per plate.

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Met Thr Glu Tyr Lys Leu Val Val Val Gly     10
ATG ACT GAG TAC AAA CTG GTG GTG GTT GGA

Ala Gly Gly Val Gly Lys Ser Ala Leu Thr     20
GCA GGT GGT GTT GGG AAA AGC GCA CTG ACA

Ile Gln Leu Ile Gln Asn His Phe Val Asp     30
ATC CAG CTA ATC CAG AAC CAC TTT GTA GAT

Glu Tyr Asp Pro Thr Ile Glu|
GAA TAT GAT CCC ACC ATA GAG|GTGAGGCCC..

                           |Asp Ser Tyr     40
..... INTRON .... CACCCCCAG|GAT TCT TAC

Arg Lys Gln Val Val Ile Asp Gly Glu Thr     50
AGA AAA CAA GTG GTT ATA GAT GGT GAA ACC

Cys Leu Leu Asp Ile Leu Asp Thr Ala Gly     60
TGT TTG TTG GAC ATA CTG GAT ACA GCT GGA

Gln Glu Glu Tyr Ser Ala Met Arg Asp Gln     70
CAA GAA GAG TAC AGT GCC ATG AGA GAC CAA

Tyr Met Arg Thr Gly Glu Gly Phe Leu Cys     80
TAC ATG AGG ACA GGC GAA GGC TTC CTC TGT

Val Phe Ala Ile Asn Asn Ser Lys Ser Phe     90
GTA TTT GCC ATC AAT AAT AGC AAG TCA TTT

Ala Asp Ile Asn Leu Tyr Ar|
GCG GAT ATT AAC CTC TAC AG|GTACTAGGA...

                          |g Glu Gln Ile   100
..... INTRON .... CGTTTTTA|G GAG CAG ATT

Lys Arg Val Lys Asp Ser Asp Asp Val Pro   110
AAG CGA GTA AAA GAC TCG GAT GAT GTA CCT

Met Val Leu Val Gly Asn Lys Cys Asp Leu   120
ATG GTG CTA GTG GGA AAC AAG TGT GAT TTG

Pro Thr Arg Thr Val Asp Thr Lys Gln Ala   130
CCA ACA AGG ACA GTT GAT ACA AAA CAA GCC

His Glu Leu Ala Lys Ser Tyr Gly Ile Pro   140
CAC GAA CTG GCC AAG AGT TAC GGG ATT CCA

Phe Ile Glu Thr Ser Ala Lys Thr Arg Gln|  150
TTC ATT GAA ACC TCA GCC AAG ACC AGA CAG|

GTATGGTA  .....  INTRON  ....  TTTA TAG|

Gly Val Glu Asp Ala Phe Tyr Thr Leu Val   160
GGT GTT GAA GAT GCT TTT TAC ACA CTG GTA

Arg Glu Ile Arg Gln Tyr Arg Met Lys Lys   170
AGA GAA ATA CGC CAG TAC CGA ATG AAA AAA

Leu Asn Ser Ser Asp Asp Gly Thr Gln Gly   180
CTC AAC AGC AGT GAT GAT GGG ACT CAG GGT

Cys Met Gly Leu Pro Cys Val Val Met       189
TGT ATG GGA TTG CCA TGT GTG GTG ATG TAA
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Fig. 2. The nucleotide sequence of the coding regions of the normal N-*ras* gene. The predicted amino acid sequence for the 189 amino acid N-*ras* protein is also presented. A 320-bp *Hind*III (see Figure 1a) fragment was found to contain the first exon and this was sequenced after cloning into *Hind*III-digested M13 mp8 and M13 mp9. A *Pvu*II site was found to cut within the second exon. The first part of this exon was subcloned into *Sma*I-digested M13 mp9 on a 1.6-kb *Pvu*II/*Pvu*II fragment and the second part was subcloned into *Sma*I/*Eco*RI-digested M13 mp8 on a 1.3-kb *Pvu*II/*Eco*RI fragment. The third exon was located within a 1.0-kb *Pst*I fragment and a *Bal*I site was found to cut within this exon. The two parts of the exon were subcloned into *Sma*I/*Pst*I-digested M13 mp8 and 9 on *Bal*I/*Pst*I fragments. The fourth exon was found within a 0.7-kb *Nde*I/*Pst*I fragment, the *Nde*I site was blunt-ended with Klenow DNA poll and the fragment cloned into *Sma*I/*Pst*I-digested M13 mp9. All fragments were sequenced using the dideoxy method on single-stranded phage clones using a 17-bp M13 primer obtained from Amersham Int. plc.

Coding sequence of the N-ras gene

Using the viral Ha- and Ki-*ras* genes as probes, Southern blot analysis of N-*ras* DNA localized the exons of the N-*ras* gene to approximately the positions shown in Figure 1a. These areas of the clone were then sequenced using the dideoxy method after subcloning into M13 vectors and the exact positions of the exons are shown in Figure 1a. By comparing these sequences with those of the other known *ras* genes, a complete exon sequence of the fetal N-*ras* gene was assembled and is presented in Figure 2. A comparison of this sequence with the sequences of c-Ki-*ras*2 and c-Ha-*ras*1 illustrates a number of interesting features. The first two coding exons (97 amino acids) of all three genes are almost identical in amino acid sequence with a maximum of five differences between any two. This is despite ~20% nucleotide replacements. The amino acid sequences of the 3rd exons show slightly greater divergence between the three genes. N-*ras* differs from c-Ha-*ras*1 at 9/53 amino acids and from c-Ki-*ras*2 at five positions (c-Ki-*ras*2 and c-Ha-*ras*1 differ by eight amino acids in this exon). It is in the 4th exon (39 amino acids) that the three genes show their greatest divergence in amino acid sequence. Compared with N-*ras*, c-Ha-*ras*1 differs at 15 positions and c-Ki-*ras*2 (exon 4B) at 22 positions. Overall, N-*ras* differs from c-Ha-*ras*1 at 28 out of 189 positions (15%) and from c-Ki-*ras*2 (exon 4B) at 32 out of 189 positions (16%).

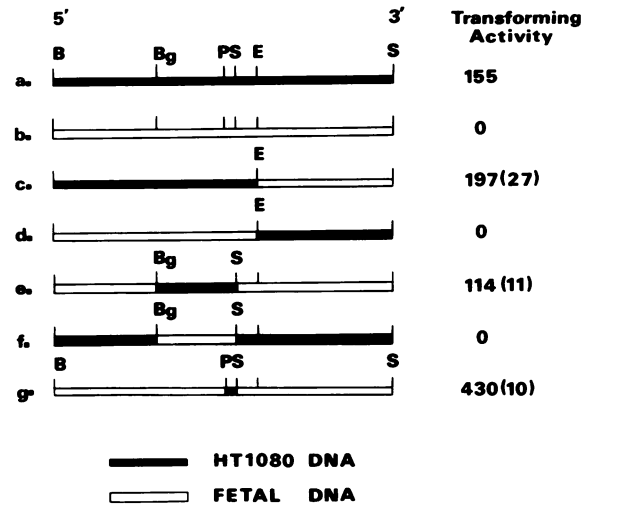

Fig. 3. Chimeric molecules constructed between the HT1080 and the fetal N-*ras* genes. In **lanes e, f and g**, chimeric plasmids were first constructed for the *Bam*HI-*Eco*RI left-hand portion of the gene. The chimeric *Bam*HI-*Eco*RI fragment was then isolated from low melting point agarose and ligated to the *Eco*RI-*Sst*I right-hand end. The DNA from this ligation mixture was used directly in the NIH/3T3 transfection assay. Figures represent the number of transformed foci obtained when a total of 1 μg of this ligation mixture was applied to NIH/3T3 cells on four plates. The figures in brackets in **c, e and g** are the number of foci obtained in the homologous recombination experiments, where 1 μg of a plasmid containing the *Bam*HI-*Eco*RI fragment and 1 μg of pN-*ras* (Fe) were mixed and applied to NIH/3T3 on one plate.

Localisation of the activating alteration in HT1080

We have already shown that the N-*ras* gene in HT1080 cells is neither rearranged nor amplified; nor does there appear to be any increase in transcription from this activated gene (Hall *et al.*, 1983). To pinpoint the exact site responsible for the activating effect, we employed a strategy that depended on *in vitro* recombination, i.e., fragments from the normal allele were replaced with reciprocal fragments from the transforming allele. Acquisition of transforming ability by the fetal gene localises the lesion to the incorporated fragment. The initial chimeric genes tested by transfection involved replacing the 8.8-kb *Eco*RI fragment of the fetal gene with the corresponding fragment from HT1080. As can be seen from Figure 3, lane c, this construct efficiently induced foci

of morphologically altered cells when tested in transfection. The reciprocal construction, the 8.8-kb fragment of fetal origin ligated to the right half of the HT1080 gene (Figure 3, lane d), showed no observable foci in transfection. Since the *Eco*RI site within the gene separates exons 1 and 2 from exons 3 and 4, this localises the lesion to the promoter, the 5′-untranslated sequences or the 1st or 2nd exon. All subsequent chimeras, therefore, involved replacing fragments within the 8.8-kb *Eco*RI half of the gene.

In parallel with the *in vitro* recombination experiments, we undertook a series of experiments utilizing *in vivo* homologous recombination. For example, it was found that although neither pAT8.8(HT) (a plasmid containing the 8.8-kb *Eco*RI fragment from HT1080) nor pN-*ras* (Fe) (Figure 1c) alone would transform NIH/3T3 cells, if these plasmids were co-transfected foci of morphologically transformed NIH/3T3 cells were obtained. We assume that a full length activated gene was created *in vivo* by homologous recombination. This phenomenon has been observed by others in gene transfer experiments (Shapira *et al.*, 1983). This procedure allows a more rapid assay but one which is an order of magnitude less efficient in the induction of foci in the transfection assay (100−400 foci/μg by mixed ligation as compared with 10−20 foci/μg using co-transfection).

Other chimeras were constructed (see Figure 3) and the smallest fragment from HT1080 that was capable of activating the normal gene was a *Pvu*II-*Sst*I fragment. The final chimera (Figure 3, lane g) contained HT1080 coding sequence between *Pvu*II and *Sst*I which comprises only a portion of the second exon from amino acids 59−97 (see Figure 2). The remainder of this construct was of fetal origin. The *Pvu*II-*Sst*I fragment isolated from HT1080 and from the fetal clone were then subcloned into M13 cloning vectors and the exon portion of the sequence determined. The sequence of the fragments was identical except for a single base change; a C to A transversion altering the 61st amino acid from glutamine in the normal allele to lysine in the HT1080 gene.

Discussion

We have previously described and partially characterized an N-*ras* gene present in the human fibrosarcoma cell line HT1080 that is capable of transforming NIH/3T3 cells (Marshall *et al.*, 1982; Hall *et al.*, 1983). Here we report the isolation of the complete, biologically active N-*ras* gene. A plasmid containing the gene, pN-*ras* (HT), transforms NIH/3T3 with high efficiency (2000 foci/μg plasmid DNA). In contrast, a full length normal N-*ras* gene, cloned from human fetal liver DNA, pN-*ras* (Fe), does not transform NIH/3T3 cells. We have mapped the 5′ and 3′ boundaries of the gene by deleting sequences from either end of the activated gene and have found that all sequences required for transformation of NIH/3T3 cells, which presumably includes the promoter and termination signals, are located between a *Pst*I site at the 5′ end and an *Sst*I sit at the 3′ end of the gene (see Figure 1a). This gives a size of 9.4 kb for the gene and confirms and extends results published by others (Shimizu *et al.*, 1983). However, as mentioned previously, the N-*ras* gene also encodes a 5.2-kb species in addition to the 2.2-kb mRNA. The larger transcript is derived from sequences running through the *Eco*RI site at the end of the 7.0-kb fragment (unpublished results).

The coding portion of the gene, localised by making use of the viral Harvey and Kirsten *ras* probes, is distributed over four exons and these have been sequenced by the dideoxy method. The location of the introns within the coding sequence is identical for c-Ha-*ras*1, c-Ki-*ras*2 and N-*ras*, though the introns themselves bear no resemblance to each other in sequence or length. Although the coding sequence and intron positions are highly conserved, the overall structures of the *ras* mRNAs differ considerably. We and others have shown that there are two N-*ras* transcripts of ~2.2 and 5.2 kb in length (Hall *et al.*, 1983; Murray *et al.*, 1983). In contrast the c-Ha-*ras*1 transcript has been identified as a 1.2-kb species (Goldfarb *et al.*, 1982) and the major c-Ki-*ras*2 transcript is much larger, at 5.5 kb (Capon *et al.*, 1983). The significance, if any, of such variable mRNA sizes is unclear but may conceivably play a role in the differential regulation of the p21 proteins.

N-*ras* codes for 189 amino acids with a predicted mol. wt. of 21 231. The amino acid sequence shows striking homology to the other two functional human *ras* genes, c-Ha-*ras*1 and c-Ki-*ras*2. When the coding sequence of the three genes is compared it is found that, overall, N-*ras* differs from c-Ha-*ras*1 at 15% of its amino acids and 27% of nucleotides (in the coding sequence) and from c-Ki-*ras*2 at 16% of its amino acids and 24% of nucleotides. However, these changes are not evenly distributed throughout the exons, in fact exons 1 and 2 have almost identical amino acid sequence (N-*ras* and c-Ha-*ras*1 differ at only four positions of the 97 comprising the 1st and 2nd exons). The third exon shows a little more divergence (N-*ras* differing from c-Ha-*ras*1 at nine positions out of 53), but it is in the 4th exon that the sequence divergence is most striking. It is likely, therefore, that the fourth exon encoded domain of the p21 protein product of the *ras* genes is involved in determining any differences there might be in action or cellular localisation of the three *ras* gene products. In this respect it may be significant that the viral Harvey p21 product undergoes some processing *in vivo* at its C terminus (Shih *et al.*, 1982).

The mechanism by which the N-*ras* gene is activated in the HT1080 cell line has been determined. We have constructed chimeric molecules betwen the HT1080 and the normal N-*ras* alleles, either *in vitro* using DNA ligase or *in vivo* using homologous recombination, and tested these for transforming activity in NIH/3T3 cells. The activating change was localized to a *Pvu*II/*Sst*I fragment which codes for amino acids 59−97 of the 2nd exon. After sequencing both the active and inactive alleles the only change detected was a C to A transversion. This results in an amino acid alteration of glutamine (CAA) in the normal gene to lysine (AAA) in the transforming allele. This fits well with other results so far obtained on the mechanism of activation of *ras* genes. Several groups have shown that the c-Ha-*ras*1 gene can be activated by an alteration at amino acid positions 12 or 61, c-Ki-*ras*2 activation has only been detected so far at position 12. An identical position 61 mutation to the one described here has also been reported to activate N-*ras* in a neuroblastoma cell line SK-N-SH (Taparowsky *et al.*, 1983). The reason for such a dramatic biological effect of a single amino acid substitution at either position 12 or 61 is obscure, though since the function of the *ras* proteins is not understood this is hardly surprising. Computer predictions have suggested that replacement of glycine at position 12 by another amino acid would have a major effect on the secondary structure at a region believed to be important to the activity of the p21 protein, the GTP binding domain (Reddy *et al.*, 1982; Wierenga and Hol,

1983). This is supported by the observation that at least several different amino acids can replace glycine and still activate the gene. We have performed similar calculations for an alteration at position 61 (Cary and Hall, unpublished information) and found no clear changes predicted in the secondary structure. On the other hand, the two different examples of 61 changes so far reported, glutamine to lysine (observed in two isolates of N-*ras*) and to leucine (in a c-Ha-*ras*1 gene) involve chemically very different amino acid residues and we would again favour a model involving some kind of conformational change (Pincus *et al.*, 1983). We would also consider the extreme high degree of homology of the 1st and 2nd exons in the three human genes, in the viral (rat-derived) genes (Dhar *et al.*, 1982; Tsuchida *et al.*, 1982) and in the yeast genes (Gallwitz *et al.*, 1983; DeFeo-Jones *et al.*, 1983) suggests very stringent requirements for the secondary and tertiary structure of this part of the molecule. Changes within these exons at other positions would be predicted therefore to produce defective *ras* gene products. Finally, since it is known that both the normal and activated (viral Harvey) rat gene products bind GTP strongly, it may be that a change in substrate specificity rather than an alteration in catalytic activity is the critical event in *ras* activation.

Materials and methods

DNA transfection

Transfections were performed as previously described (Wigler *et al.*, 1979). The NIH/3T3 cells were seeded at 1.5 x 10^5 cells/60 mm plate in Dulbecco's modified Eagles medium plus 10% calf serum one day before transfection. Between 0.1 and 1 μg of plasmid DNA or of the mixed ligations was transfected into NIH/3T3 cells as a calcium phosphate precipitate together with 20 μg/plate of mouse carrier DNA. In the *in vivo* recombination experiments 1 μg of each plasmid was used. After transfection the cells were maintained in Dulbecco's/Eagles medium and 5% calf serum. The cells were medium changed every 3–4 days for the 14-day duration of the experiment. Plates were examined for foci of morphologically altered cells from day 12.

Cloning of the normal and the transforming gene

HT1080. DNA was isolated from a second-round HT1080 transfectant, digested with *Hind*III and used to construct a library in the phage vector L47.1 (Loenen and Brammar, 1980). 100 000 recombinants were screened with nick-translated probe A and four positive phages were isolated. One of these was examined further and found to contain an 11-kb *Hind*III fragment, the first 1 kb of which overlapped with the previously isolated 8.8-kb *Eco*RI fragment. To construct a plasmid containing a full length N-*ras* gene from HT1080, a three-way ligation was set up using the large *Sst*I/*Bam*HI fragment from the plasmid pHLTR (Chang *et al.*, 1982), a *Bam*HI/*Eco*RI fragment from pAT8.8 (HT) and an *Eco*RI/*Sst*I fragment from the 11-kb *Hind*III fragment described above. This yielded a biologically active plasmid pN-*ras* (HT) (see Figure 1b). pN-*ras* (HT) is large (~17 kb) and contains at least three repetitive elements; to ensure deletions did not occur during propagation of this plasmid in *Escherichia coli*, the host strain ED8767 (rec A^-) (Murray *et al.*, 1977) was used.

Normal. DNA was isolated from fetal liver, partially digested with *Eco*RI and fractionated on a 5–20% potassium acetate gradients. Fractions were collected and aliquots of each were screened, using Southern blots, with probes A and E (Figure 1a) separately. Those fractions of ~16 kb, that were found to contain both the 8.8-kb and 7.0-kb fragments were used to construct a library in L47.1. Recombinants were selected on a P2 lysogen and screened with probe A. A phage clone was identified which contained a 16-kb insert and which had an identical restriction map to the N-*ras* gene isolated from HT1080. The large (7.8 kb) *Bam*HI/*Eco*RI fragment from this clone (derived from the 8.8-kb *Eco*RI fragment) was subcloned into pAT153. The 7.0-kb *Eco*RI fragment, also from the fetal N-*ras* clone, was then subcloned into the *Eco*RI site of this plasmid, the correct orientation was identified and a plasmid pN-*ras* (Fe) containing a full length normal N-*ras* gene was obtained (see Figure 1c). pN-*ras* (Fe) was also maintained in the host strain ED8767.

Chimera constructions

After digestion with the appropriate enzymes, fragments were isolated from low melting point agarose gels by phenol extraction at 65°C. Mixtures of purified fragments were ligated overnight with T4 DNA ligase at 15°C. This DNA was then added directly to NIH/3T3 cells as described.

Sequencing

Fragments to be sequenced were cloned into the replicative form of one of the phage vectors M13 mp8 or mp9. These were transfected into JM101 cells and recombinants identified as colourless plaques on X-gal-containing plates. Single-stranded phage containing the insert were then isolated and sequenced by the dideoxy method (Sanger *et al.*, 1977).

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