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Structure and expression of amplified cKi-*ras* gene sequences in Y1 mouse adrenal tumor cells

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A recombinant library of double minute chromosomal DNA, enriched in specific sequences that are amplified in Y1 mouse adrenal tumor cells, was used as a source of material to explore the structure and expression of amplified cKi-*ras* genes in these cells. From DNA sequence analysis of these cloned fragments, we found no evidence for the presence of point mutations previously demonstrated to be associated with activation of the transforming potential of *ras* genes. A comparison of the mouse gene with that of the homologous human cKi-*ras*2 gene reveals 94% nucleotide sequence homology within the coding regions and 97% homology for the predicted amino acid composition. Like the human gene, the mouse cKi-*ras* gene contains alternative 3′ coding exons. Blot hybridization analyses of RNA revealed a preferential utilization of the more 3′ of the two fourth coding exons in the generation of Y1 cKi-*ras* transcripts.

Key words: double minutes/gene amplification/oncogenes/*ras* genes/transformation

Introduction

A growing number of mammalian tumor cells and transformed cell lines have been shown to contain amplified copies of genes (proto-oncogenes) related to viral transforming genes (oncogenes) (for recent reviews, see Land *et al.*, 1983; George, 1984). The resulting enhanced expression of such cellular sequences has been proposed to play a direct role in the initiation, progression and/or maintenance of some tumors. To understand the mechanisms by which gene amplification might contribute to cellular transformation, it is important to know whether such events involve overexpression of an otherwise normal gene product, or whether critical alterations in gene sequence also have occurred. We have explored this question using Y1 mouse adrenal tumor cells in which a cKi-*ras* gene is amplified 30- to 60-fold (Schwab *et al.*, 1983). In the Y1 cells, the amplified cKi-*ras* genes are located on either of two classes of chromosomal abnormality: double minute chromatin bodies (Y1-DM cells), or homogeneously staining chromosomal regions (Y1-HSR cells) (George and Powers, 1981, 1982).

The cellular cKi-*ras* gene is a homologue of the transforming gene (vKi-*ras*) of a rat-derived virus, the Kirsten murine sarcoma virus (KiMuSV) (Kirsten and Mayer, 1967; Ellis *et al.*, 1981; Chang *et al.*, 1982a). Members of the highly conserved *ras* gene family, which includes the Ki-, Ha- and N-*ras* genes, encode closely related, yet distinct, guanine nucleotide-binding proteins of ~21 000 daltons, termed p21 proteins (Ellis *et al.*, 1981; Papageorge *et al.*, 1982; Taparowsky *et al.*, 1983). Based on their ability to induce tumorigenic transformation of recipient cells in DNA-mediated gene-transfer experiments, members of the *ras* family have been been implicated as transforming genes in a large number of human and rodent tumor cells (Der *et al.*, 1982; Pulciani *et al.*, 1982; Eva *et al.*, 1983; Shimizu *et al.*, 1983a; Hall *et al.*, 1983). In such tumor cells, the *ras* genes generally have not been amplified or overexpressed. Several studies have demonstrated that 'activation' of the transforming potential of *ras* genes isolated from tumor cells resulted from single point mutations affecting amino acid 12 or 61 of the respective *ras* proteins (Reddy *et al.*, 1982; Tabin *et al.*, 1982; Taparowsky *et al.*, 1982, 1983; Capon *et al.*, 1983a; McGrath *et al.*, 1983; Shimizu *et al.*, 1983b; Yuasa *et al.*, 1983). Mutations that specify amino acid substitutions at positions 13, 59 or 63 also can activate the transforming potential of *ras* genes (Fasano *et al.*, 1984).

We report here analyses of Y1 cKi-*ras* sequences that, by virtue of their location on DMs, represent amplified copies of the cellular gene. These DNA fragments were isolated from a subgenomic library constructed using DNA obtained from a chromosomal fraction enriched in DMs (Schwab *et al.*, 1983). In previous work, we demonstrated that the DM chromosomal fraction and the library constructed from it are highly enriched in specific nucleotide sequences that are amplified in the Y1 cells, including the cKi-*ras* gene (George and Powers, 1981; Schwab *et al.*, 1983). The DM-enriched fraction does not contain detectable levels of sequences from other cellular genes, such as cHa-*ras* or c-*myc*, that are not amplified in the Y1 cells (Schwab *et al.*, 1983; our unpublished data). Here we present data consistent with the conclusion that some, if not all, of the amplified cKi-*ras* genes have a normal coding sequence. In addition we have found that the mouse cKi-*ras* gene, like the homologous human cKi-*ras*2 gene (Capon *et al.*, 1983b; McGrath *et al.*, 1983; Shimizu *et al.*, 1983b), contains alternative fourth coding exons, providing the potential to encode two distinct p21 cKi-*ras* products. These two 3′-coding exons are not equally represented among processed cKi-*ras* transcripts in the Y1 cells.

Results

Gene organization

To isolate recombinant clones derived from amplified copies of the cKi-*ras* gene, we screened a phage library of Y1 DM-DNA fragments. Positive plaques were identified by hybridization to vKi-*ras* clone pHiHi3, which contains sequences homologous to exons 1, 2, 3 and 4A of the cellular gene (Tsuchida *et al.*, 1982). The library was also screened with cDNA clones isolated from a Y1-DM cDNA library, one of which (cYki2) has been shown to be homologous to exons 3 and 4B of the human gene (George *et al.*, 1984). 250 of 30 000 plaques screened with pHiHi3 produced a hybridization signal. The relatively large number of positive plaques detected provides additional evidence that the Y1 DM-DNA library is highly enriched in cKi-*ras* sequences. In Southern blot analysis, hybridization of vKi-*ras* probe pHiHi3 to *Eco*RI-digested mouse genomic DNA reveals fragments

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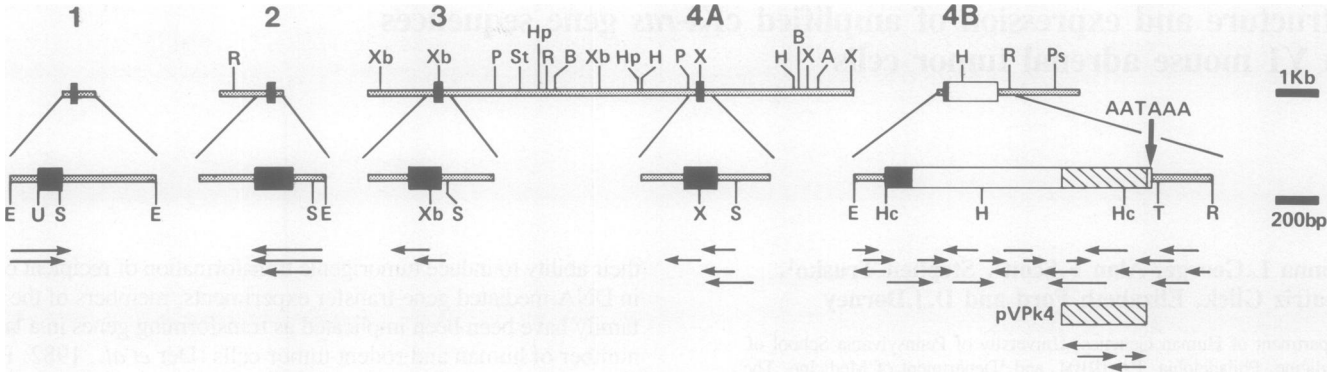

Fig. 1. Restriction maps and strategy for DNA sequence analysis of DM-associated mouse cKi-*ras* gene. Genomic *Eco*RI fragments having homology to the various Ki-*ras* probes described in the text are shown. Potential coding regions are indicated as solid boxes and are also presented on an expanded scale with sequencing strategy (arrows). The relative position of cDNA clone pVPk4 (hatched) within the 3′-non-translated region (open box) is also indicated. Abbreviations for restriction endonucleases: A, *Ava*I; B, *Bgl*II; E, *Eco*RI; H, *Hind*III; Hc, *Hinc*II; Hp, *Hpa*I; P, *Pvu*II; Ps, *Pst*I; S, *Sau*3A; St, *Sst*I; U, *Stu*I; X, *Xho*II; Xb, *Xba*I. Not all sites are shown for a few endonucleases.

~11.5, 8.5, 1.5 and 0.5 kb in size (Ellis *et al.*, 1981; Schwab *et al.*, 1983). A 3.4-kb *Eco*RI fragment is revealed by hybridization to cDNA clones cYki2 and pVPk4 (George *et al.*, 1984). All of these genomic fragments are amplified in the Y1 cells.

We identified representative phage particles containing all of these *Eco*RI fragments, each of which was subcloned into pBR322. Restriction maps of these fragments were constructed and the 5′→3′ orientation determined by DNA sequence analysis, as indicated in Figure 1. Based on restriction endonuclease analyses of the cloned fragments and of genomic DNA samples we estimate that the mouse cKi-*ras* gene, like the homologous human gene, spans a region >40 kb in size. Coding regions were identified by blot hybridization experiments and by comparing results of DNA sequence analyses with the published sequences of the vKi-*ras* (Tsuchida *et al.*, 1982) and human cKi-*ras*2 genes (Capon *et al.*, 1983b; McGrath *et al.*, 1983; Shimizu *et al.*, 1983b). This allowed us to assign the various coding exons to each of the *Eco*RI genomic fragments isolated. (The 8.5-kb fragment contains sequences homologous to a region 5′ of the first coding exon, and will not be described in this communication.) The nucleotide sequences obtained for the coding exons and their immediate flanking regions are presented in Figure 2. Consensus donor and acceptor splice signals (Mount, 1982) were found at intron/exon boundaries.

A comparison of the predicted amino acid composition of the DM-associated cKi-*ras* sequences with those of the rat-derived vKi-*ras* gene and the human cKi-*ras*2 gene is presented in Figure 3. As stated previously, a number of studies have demonstrated that activation of the transforming potential of *ras* genes can be correlated with nucleotide changes that result in single amino acid substitutions at positions 12, 13, 59, 61 or 63. The vKi-*ras* gene differs from the normal human gene at amino acids 12 and 59. It should be noted that at all five of these positions, the predicted amino acid composition of the amplified cKi-*ras* is identical to that of the normal human gene.

There is a very high degree of DNA sequence and amino acid homology among these three Ki-*ras* genes (Figure 3). Between the Y1 mouse and vKi-*ras* genes, for sequences contained within exons 1, 2, 3 and 4A, there are only 15 nucleotide differences (15/567 or 2.6%), resulting in four amino acid differences (4/189 or 2.1%). Between the mouse and human genes, there are only 41 differences among the 681 nucleotides (6.0%) comprising the five potential coding exons. These result in only six amino acid differences (6/227 or 2.6%).

The 3′-non-translated region

The mouse cKi-*ras* gene encodes two major mRNA species, ~5.2 and 2.0 kb in size (Schwab *et al.*, 1983; George *et al.*, 1984; see Figure 4), both of which have been translated *in vitro* to yield p21$^{cKi\text{-}ras}$ products (Ellis *et al.*, 1982). A minor RNA species of ~1.2 kb in size is also present in the Y1 cells (Schwab *et al.*, 1983). Our DNA sequence analysis of the 3′-non-translated portion of the Y1 gene revealed a potential polyadenylation signal, AATAAA (Proudfoot and Brownlee, 1974), located ~1100 bp downstream from the termination codon in exon 4B (Figure 2). The presence of this sequence as a potential signal for polyadenylation in the derivation of the 2-kb mRNA was supported by DNA sequence analysis of a cKi-*ras* cDNA clone (pVPk4) that we had previously isolated from a Y1 cDNA library. As indicated in Figure 2, this cDNA sequence terminates at a position just 3 bp upstream of the AATAAA. No other AATAAA sequence was detected upstream of this site that might account for the minor 1.2-kb RNA species. However, the hexanucleotides ACTAAA and AATGAA were found 505 and 604 bp, respectively, downstream of the exon 4B termination codon and might function as weak polyadenylation signals in the generation of the smaller message.

A comparison of the mouse and human cKi-*ras* genes in the flanking region immediately 3′ of exon 4B revealed a homology of ~79% (159 of 201 bp, with gaps counted as single events). The relatively high degree of conserved sequence homology may suggest a functional role for this region.

Prevalence of exon 4B transcripts

Because we found that the mouse cKi-*ras* gene, like the human gene, contains alternative fourth coding exons, we ascertained whether both exons 4A and 4B are equally represented in the procesed RNA transcripts of the Y1 cells. Northern blots were prepared using RNA from the Y1 cells, as well as from a KiMuSV-infected, BALB-3T3 cell line, termed K-BALB. The RNA blots were hybridized to M13-derived probes specific for exon 4A or exon 4B. For the Y1 cells, transcripts containing 4B are in much greater abundance than those with exon 4A (Figure 4). Based on the intensity of the hybridization signals we estimate that exon 4B-containing transcripts are at least 25 times more abundant than exon 4A-containing transcripts. However, cKi-*ras* mRNAs with exon 4A sequences are more abundant in the Y1 cells compared with their levels in cells that do not overexpress cKi-*ras* (data not shown). In contrast, both

Fig. 2. Nucleotide sequence and predicted amino acid composition of Y1 cKi-*ras* coding exons and their immediate flanking regions. Sequence information is based on the strategy described in Figure 1. Intron/exon junctions are indicated by arrows. Numbering begins at the first codon in exon 1. The region of homology to cDNA pVPk4 is indicated, as is a putative polyadenylation signal AATAAA (++++++).

```
                1         10         20         30         40         50
Y1 Mouse        MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGET
Human           --------------------------------------------------
vKi-ras         ----------S---------------------Q-------------

                51         60         70         80         90        100
Y1 Mouse        CLLDILDTAGQEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIHHYREQI
Human           --------------------------------------------------
vKi-ras         --------T-------------------------------------L

                101        110        120        130        140        150
Y1 Mouse        KRVKDSEDVPMVLVGNKCDLPSRTVDTKQAQELARSYGIPFIETSAKTRQ
Human           ------------------------------D-------------------
vKi-ras         --------------------------------------------------

                Exon 4A
                151        160        170        180
Y1 Mouse        RVEDAFYTLVREIRQYRLKKISKEEKTPGCVKIKKCVIM
Human           ----------------------------------I--
vKi-ras         ----------------------------------------

                Exon 4B
                151        160        170        180
Y1 Mouse        GVDDAFYTLVREIRKHKEKMSKDGKKKKKKSRTRCTVM
Human           ------------------------------K-K-VI-
```

Fig. 3. Comparison of the predicted amino acid sequences of the Y1 mouse, human and viral Ki-*ras* genes. The human and viral sequences were taken from published information (as described in the text). A dashed line indicates that the amino acid is the same as that predicted for the mouse gene. The standard one-letter code for amino acids was used.

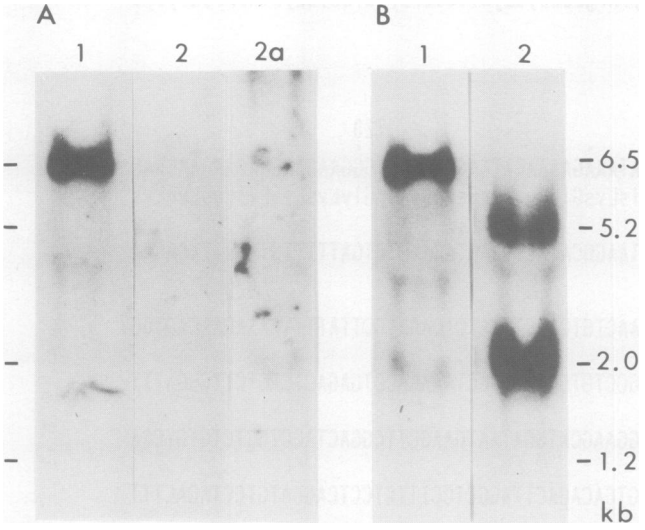

Fig. 4. Filter hybridization analysis of cKi-*ras* transcripts. Samples of total cellular RNA (20 μg) from K-BALB cells **(lane 1)** or from Y1 cells **(lane 2)** were transferred to nitrocellulose filters and hybridized to ^{32}P-labeled M13 probes derived from: **(A)** exon 4A (*Sau*3A fragment including 100 nucleotides of coding region and 180 nucleotides of immediate 3′-flanking material); **(B)** exon 4B (*Eco*RI-*Hind*III fragment including 114 nucleotides of coding region and 490 nucleotides of immediate 3′- and 5′-flanking material). Exposure time, 1 day. In **(A), lane 2a** is a longer exposure (3 days) of **lane 2**.

exon 4A and exon 4B are equally represented in the RNA (~6.5 kb in size) transcribed from the KiMuSV genome in the K-BALB cells (Figure 4). Although exon 4B-derived sequences are present in the viral genome, they apparently are not translated because of their location downstream of the exon 4A-derived termination codon (McGrath *et al.*, 1983; Shimizu *et al.*, 1983b).

Discussion

We have isolated and characterized recombinant clones obtained from amplified copies of the mouse cKi-*ras* proto-oncogene that are present on DMs in Y1 adrenal tumor cells. From DNA sequence analysis of the Y1 material we found no evidence for the presence of point mutations previously associated with activation of the transforming ability of viral or cellular members of the *ras* gene family. DNA sequence information on a cKi-*ras* gene isolated from normal mouse cells is currently available only for exon 1 (Guerrero *et al.*, 1984). In exon 1, the nucleotide sequence of the Y1-derived material is identical to that derived from the normal gene. Our data demonstrate a very high degree (>95%) of nucleotide sequence homology between the Y1 mouse and human cKi-*ras* genes. Only six differences were observed in the predicted amino acid sequence between the Y1 product and that encoded by the normal human cKi-*ras*2 gene. Five of these represent conservative amino acid replacements. Only at codon 187, in exon 4B, is there a non-conservative change (Thr for Val). Results obtained in a recent study indicate that a Thr at position 187 has no obvious effect on the biological activity of a Ha-*ras* product (Willumsen *et al.*, 1984). Ascertaining whether the function of the cKi-*ras* protein might be affected by any of the other five differences in predicted amino acid composition between the Y1 product and that of normal human gene must await more information, including additional DNA sequence data on the gene isolated from normal mouse cells.

The data presented here are consistent with the idea that it is a 'normal' cKi-*ras* gene that has been amplified in the Y1 cells. However, because it is not practical to determine the DNA sequence of all 30–60 copies of the amplified gene, we cannot rule out completely the possibility that one or a few copies of the gene could have a different sequence. That the tumorigenic properties of the Y1 cells might be associated with enhanced expression of a normal cKi-*ras* gene is supported by other data. Studies employing DNA-mediated gene transfer techniques have demonstrated that a normal allele of the cHa-*ras* or cKi-*ras* gene can transform recipient cells, if such a cloned gene is ligated to a high efficiency retroviral promoter to achieve enhanced expression (Chang *et al.*, 1982b; McCoy *et al.*, 1984). Other results have also demonstrated a positive correlation between high levels of expression of a normal *ras* gene and transforming activity (Taparowsky *et al.*, 1982; Samid *et al.*, 1984). Understanding the way in which such *ras* gene overexpression might mediate cellular transforming events awaits more information about the role these genes play in normal growth or development.

The presence in the cKi-*ras* gene of alternative fourth coding exons provides the potential to produce different p21 proteins. These could be distinguished by the presence of a more highly basic carboxy-terminal region if exon 4B sequences are translated. Although the factors involved in the choice of splicing pathways for the cKi-*ras* gene are not understood, RNA blot analyses reveal that most transcripts lack exon 4A (Figure 4). The sequence of a cDNA clone that we previously isolated from a Y1 cDNA library (George *et al.*, 1984) also reflects a splicing event that joined exon 3 to exon 4B. A greater abundance of exon 4B-specific transcripts, compared with exon 4A-containing ones, has to date been detected in a variety of human and mouse tumor cell lines and normal tissues (Capon *et al.*, 1983b; our unpublished results). This includes human tumor cells in which cKi-*ras* genes have been activated by point mutations and are not overexpressed. Control of the relative abundance of exon 4A- and exon 4B-specific mRNAs may be an important factor in normal or aberrant processes of growth and development. Although an earlier study showed that cKi-*ras* transcripts are expressed at fairly similar levels in mouse embryos, extraembryonic tissues and a variety of post-natal tissues (Müller *et al.*, 1983), those experiments were carried out with molecular probes that would not have distinguished between RNAs derived from exon 4A or exon 4B sequences. It might be useful, therefore, to re-examine

this question to ascertain if there are tissue-specific or developmentally-related differences in the use of the 3'-coding exons of the cKi-*ras* gene.

Materials and methods

Cell lines

The origin and characterization of the Y1 cell lines have been reported (George and Powers, 1981, 1982). K-BALB, a KiMuSV-infected, non-producer, BALB/3T3 cell line (Aaronson and Weaver, 1971) was kindly provided by K. Heubner (Wistar Institute, Philadelphia, PA).

Recombinant plasmids and bacteriophage

Construction of a recombinant library of Y1 DM-DNA fragments in the lambda vector Charon 4A has been detailed (George and Powers, 1981). Phage were screened using the plaque-hybridization procedure of Benton and Davis (1977) and nitrocellulose filters washed under stringent conditions of 0.1 x NaCl/Cit, 0.1% SDS at 65°C. (1 x NaCl/Cit is 150 mm NaCl, 15 mM sodium citrate, pH 7.0.) Subcloning of DNA fragments into the plasmid pBR322 and isolation of plasmid and bacteriophage DNA followed published protocols (George and Powers, 1981). The plasmid pHiHi3 contains a 1-kb insert of vKi-*ras* sequences in the *Eco*RI site of pBR322 (Ellis *et al.*, 1981). Construction of a cDNA library from Y1-DM poly(A)$^+$ RNA and isolation of cKi-*ras* cDNA clones cYKi2 and pVPk4 have been described (George *et al.*, 1984). The 197-bp insert of cYKi2 contains sequences derived from exons 3 and 4B.

Sequencing of nucleic acids

Nucleotide sequences were determined by the method of Sanger *et al.* (1979) after subcloning of appropriate restriction endonuclease-generated DNA fragments into phage M13 (Messing and Vieira, 1982). A sequence was usually confirmed by analysis of both strands and/or of one strand from different clones.

RNA isolation and blot hybridization

Samples of total cellular RNA were prepared with guanidine-hydrochloride, denatured, subjected to electrophoresis through 1.2% agarose, 6% formaldehyde gels, and transferred to nitrocellulose in 10 x NaCl/Cit using protocols previously detailed (George *et al.*, 1984). After hybridization, the RNA blots were washed in 0.1 − 0.3 x NaCl/Cit, 0.1% SDS, at 55°C.

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