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Identification of a provirally activated c-Ha-*ras* oncogene in an avian nephroblastoma via a novel procedure: cDNA cloning of a chimaeric viral-host transcript

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Retroviruses without oncogenes often exert their neoplastic potential as insertional mutagens of cellular proto-oncogenes. This may be associated with the production of chimaeric viral-host transcripts; in these cases, activated cellular genes can be identified by obtaining cDNA clones of bipartite RNAs. This approach was used in the analysis of chicken nephroblastomas induced by myeloblastosis-associated virus (MAV). One tumor contained a novel mRNA species initiated within a MAV LTR. cDNA cloning revealed that this mRNA encodes a protein of 189 amino acids, identical to that of normal human Ha-*ras*-1 at 185 positions, including positions implicated in oncogenic activation of *ras* proto-oncogenes; there are no differences between the coding sequences of presumably normal Ha-*ras* cDNA clones from chicken lymphoma RNA and the tumor-derived cDNAs. The chimaeric mRNA in the nephroblastoma is at least 25-fold more abundant than c-Ha-*ras* mRNA in normal kidney tissue, and a 21-kd *ras*-related protein is present in relatively large amounts in the tumor. We conclude that a quantitative change in c-Ha-*ras* gene expression results from an upstream insertion mutation and presumably contributes to tumorigenesis in this single case. Little or no increase in c-Ha-*ras* RNA or protein was observed in other nephroblastomas.

Key words: myeloblastosis-associated virus-2(N)/insertional mutation

Introduction

Retroviruses lacking oncogenes derived from conserved cellular genes are often tumorigenic. Some of these viruses initiate the neoplastic process by acting as insertional mutagens of proto-oncogenes (Varmus, 1984). This phenomenon establishes that proto-oncogenes participate in tumorigenesis, provides examples of gene activation in vertebrate cells and opens avenues for the identification of new proto-oncogenes.

To date two approaches have been used to identify proto-oncogenes susceptible to insertional activation. The first uses hybridization probes homologous to known oncogenes to seek elevated expression and/or genomic rearrangements in tumors (Hayward *et al.*, 1981). This approach has revealed that insertional activation of the proto-oncogene c-*myc* features in many different types of tumors induced by a variety of non-defective retroviruses (Noori-Daloii *et al.*, 1981; Corcoran *et al.*, 1984; Neil *et al.*, 1984; Steffen, 1984; Li *et al.*, 1984). Insertional mutations of c-*erb*-B and c-*mos* have been deduced in a similar manner (Rechavi *et al.*, 1982; Fung *et al.*, 1983; Canaani *et al.*, 1983).

These results suggested a second approach to identify crucial insertional lesions. If activation of a certain gene is pivotal in tumor formation, then independent tumors of the same type will exhibit proviruses integrated in the same region of the genome. To show this, cellular sequences retrieved by virtue of physical linkage to a provirus are used to seek genomic rearrangements in other tumors. The process whereby a gene is cloned via its linkage to a mobile genetic element was first used in the isolation of the *white* locus from *Drosophila melanogaster* (Bingham *et al.*, 1981) and has been termed 'transposon tagging'. Several putative proto-oncogenes, including *int*-1 (Nusse and Varmus, 1982), *int*-2 (Peters *et al.*, 1983), *Mlvi*-1 (Tsichlis *et al.*, 1983), *pim*1 (Cuypers *et al.*, 1984) and others (Lemay and Jolicoeur, 1984; Graham *et al.*, 1985) have been identified using this type of protocol.

With a view to defining novel targets for insertional mutation, we have been investigating kidney tumors induced by the avian retrovirus, myeloblastosis-associated virus (MAV) (Smith and Moscovici, 1969). These nephroblastomas are particularly interesting as they are histopathologically similar to human Wilms' tumor (Ishiguro *et al.*, 1962; Heine *et al.*, 1962; Watts and Smith, 1980). We have sought rearrangements in nephroblastoma DNA using probes for known oncogenes and the 'transposon tagging' technique. Both approaches have yielded negative results. A third approach has been more fruitful. Analysis of nephroblastoma RNA revealed aberrant, viral transcripts in a subset of tumors, consistent with provirally initiated transcription of host DNA sequences. Cloning of cDNA generated from one such chimaeric transcript identified the c-Ha-*ras* gene as a target for proviral activation. Nucleotide sequences reveal that the transcript encodes a 21-kd protein apparently lacking the missense mutations encountered in transforming *ras* genes. We deduce that an insertion mutation dictates a qualitative change in *ras* gene expression; however, some pecularity of the insertional mutation has prevented direct detection of the c-Ha-*ras*-linked MAV provirus.

Results

MAV-induced nephroblastomas are cloned outgrowths

Figure 1 shows the physical structure of a MAV provirus and the map positions of molecular hybridization probes used in this paper. The approximate composition of the two major transcripts produced as a normal consequence of productive infection are also shown. Analysis of host viral junction fragments derived from tumor DNA was performed to determine whether the tumors in our collection contained clonal cell populations, as predicted if the tumors were induced by insertion mutations. MAV contains at least two *Eco*RI sites in the central region of the provirus (Souza and Baluda, 1980; Figure 1). Hence in a digest of genomic DNA, each MAV provirus will generate two viral-host DNA restriction fragments that may be detected using LTR-specific hybridization probes (Figure 2A). If proviruses are integrated randomly into the chicken genome in a non-clonal cell

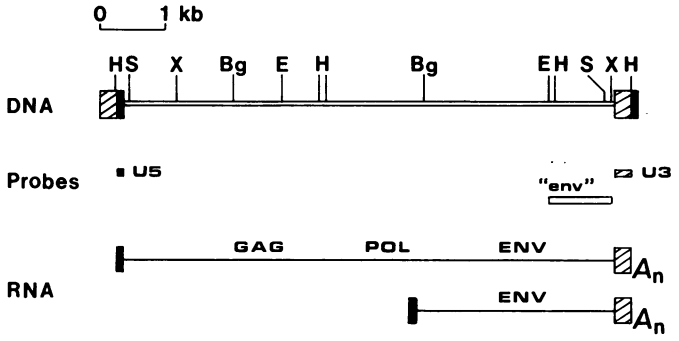

Fig. 1. Structure of a MAV-type provirus and MAV mRNA. The LTRs are shown as boxes with the U3 region striped and the U5 region in black. The positions of hybridization probes are shown underneath the provirus. The restriction map has been compiled from Bergmann *et al.* (1980), Souza and Baluda (1980) and the unpublished data of D.Westaway. Therefore the diagram represents a consensus MAV provirus. The *Hind*III site 1.2 kb from the 3′ end of the genome may not be present in all MAV isolates (Bergmann *et al.*, 1980). The U5 probe has been described previously (Payne *et al.*, 1981). A 0.85-kb *Eco*RI-*Xho*I fragment, 'env', specific for 3′ regions of the MAV genome, was excised from a molecular clone of the MAV provirus in nephroblastoma 880 (D.Westaway, unpublished). The U3-specific probe is a 0.3-kb *Xho*I-*Hind*III fragment from cloned AMV DNA (Klempnauer *et al.*, 1982). The approximate coordinates of the two major proviral transcripts are shown. 'A_n' designates the poly(A) tail. Bg = *Bgl*II; E = *Eco*RI; H = *Hind*III, S = *Sac*I; X = *Xho*I.

population, then a large number of variously sized *Eco*RI junction fragments will be generated; this will be manifest as a smear of hybridization in a Southern transfer analysis (Southern, 1975). However, if the tissue sample contains clonal populations of infected cells, then discrete junction fragment bands will be seen. (This method for assessing clonality in retrovirus-infected cell populations has been discussed extensively elsewhere, e.g. Cohen *et al.*, 1979; Neel *et al.*, 1981; Payne *et al.*, 1981).

High mol. wt. DNAs were digested to completion, electrophoresed on an agarose gel, transferred to a nitrocellulose filter and hybridized with a radiolabeled restriction fragment specific for the U3 domain of the long-terminal repeat (LTR) (Gonda *et al.*, 1981; Klempnauer *et al.*, 1982) (Figure 2B). This reagent hybridizes only with the nucleic acid sequences of viruses derived from the BAI-A stock of AMV. Thus uninfected chicken DNA gives no signal with this probe (lane 1). Non-neoplastic tissue from bird 1638 gives a smear of hybridization (lane 2), indicating that these cells are chronically infected with MAV proviruses. Nephroblastoma 1638 DNA gives a similar signal except that bands of intense hybridization, characteristic of clonal cell populations, are also present. Discrete bands of hybridization are seen in other nephroblastoma DNAs (Figure 2B, lanes 4 – 10).

Analogous results have been obtained for all the well-documented instances of insertion mutations by retroviruses without oncogenes. We therefore attempted to define a target for insertional lesions. However, analyses of tumor DNAs with a number of *onc*-specific probes, including *myc, src, erb*-A and -B and *yes*, failed to reveal any novel tumor-specific restriction fragments (data not shown). We then performed a 'transposon tagging' experiment by cloning the single provirus and its flanking host DNA from tumor 880. However, 18 other tumors tested did not exhibit rearrangements in a 30-kb interval flanking the unique provirus (D.Westaway, unpublished; Nusse *et al.*, 1984a). These negative results do not *per se* exclude the insertional mutation hypothesis; they can be rationalized by multiple and/or large genetic targets for mutations that predispose to tumor formation.

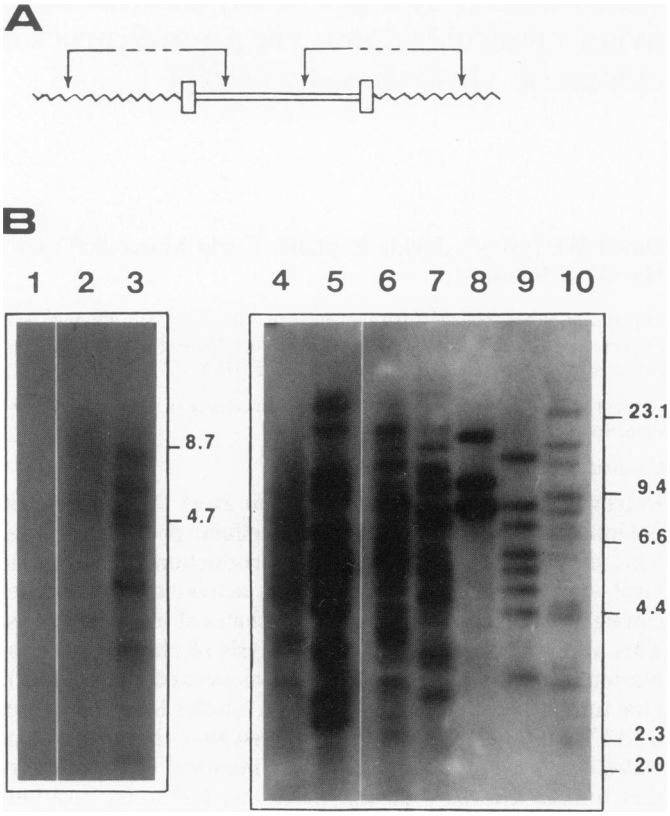

Fig. 2. Southern blot analysis of MAV-induced nephroblastomas. **Panel A** shows the origin of the junction restriction fragments which are detected with LTR-specific hybridization probes. A provirus is shown with the LTRs designated by open rectangles. Host DNA sequences are denoted by a zig-zagged line, and restriction endonuclease cleavage sites by arrows. **Panel B** shows the Southern transfer analyses with *Eco*RI (**lanes 1, 2, 3**) and *Bgl*II (**lanes 4 – 10**) digests of genomic DNA electrophoresed on agarose gels and transferred to nitrocellulose. Both of the restriction enzymes cleave within the central region of MAV proviruses. Filters were hybridized with a U3-specific probe. Positions of 8.7- and 4.7-kb size markers were deduced by re-hybridization of the filter with radiolabeled clone 34 (see below). A *Hind*III digest of phage lambda DNA was co-electrophoresed as a size marker, for the right-hand filter. **Lane 1**: uninfected chicken liver DNA. **Lane 2**: control (heart) DNA from bird 1638. **Lane 3**: nephroblastoma 1638 DNA. **Lanes 4 – 10**: nephroblastomas 1620, 1641, 1643, 1646, 636/1, 854, 193.

Identification of aberrant proviral transcripts in two nephroblastomas

We sought another feature of some insertional mutations: the production of aberrant virus-related transcripts. All of the tumors in our collection harbor the conventional 7.8- and 3.3-kb species of viral RNA (Figures 1 and 3). However, at least two tumors, 890 (Figure 3, lanes 1 and 2) and 1638 (lanes 3 and 4), contain additional, smaller RNA species which are detected by a virus-specific hybridization probe. The small RNAs, 1.2 kb (lane 3) and 1.4 kb (lane 1), were detected with a U5-specific probe but not with a U3-specific probe (lanes 2 and 4), implying that these transcripts are not terminated via a proviral polyadenylation signal. Similar transcripts containing only the U5 sequences for proviral DNA are frequently generated from provirally activated c-*myc* loci in which the proviral LTR and c-*myc* share the same transcriptional orientation (Hayward *et al.*, 1981; Payne *et al.*, 1982).

We sought to establish the chimaeric nature of the 1.2- and

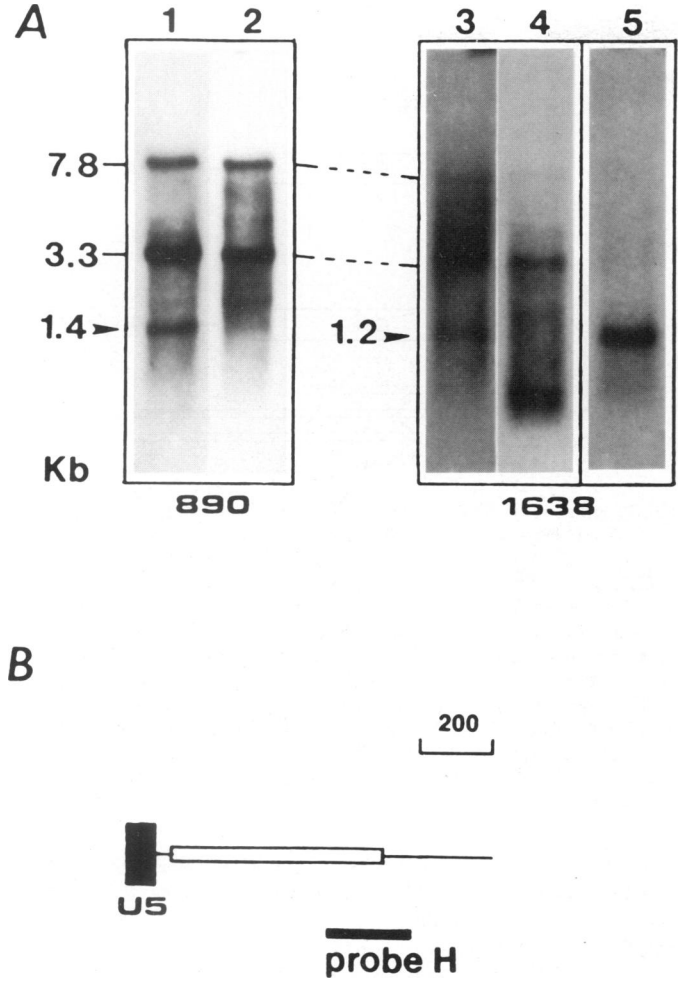

Fig. 3. Two nephroblastomas harbor aberrant viral transcripts. Polyadenylated RNA (5 μg) was isolated from tumor 890 (**lanes 1** and **2**) and 1638 (**lanes 3–5**), subjected to electrophoresis, transferred to nitrocellulose and annealed sequentially with probes for U5 (**lanes 1** and **3**) and U3 (**lanes 2** and **4**). **Lane 5** shows annealing to probe H from cDNA clone 34 (see below). Very small RNA species detected in **lane 4**) reflect slight degradation of this RNA sample. These species are not apparent in **lane 3**, presumably because degraded RNAs containing U5 sequences are not selected by oligo(dT) chromatography. Radiolabeled *Hind*III fragments of phage lambda DNA were co-electrophoresed as size markers. Approximate sizes are shown in kilobases. **Panel B** shows the structure of cDNA clone 34 obtained from nephroblastoma 1638 RNA. U5-related sequences are shown in black. The open rectangle indicates an open reading frame (see Figure 8). The 341-bp *Hinf*I fragment 'probe H' extends from nucleotides 313–654 of clone 34 (Figure 8) and is shown by a solid bar. The scale bar represents a length of 200 nucleotides.

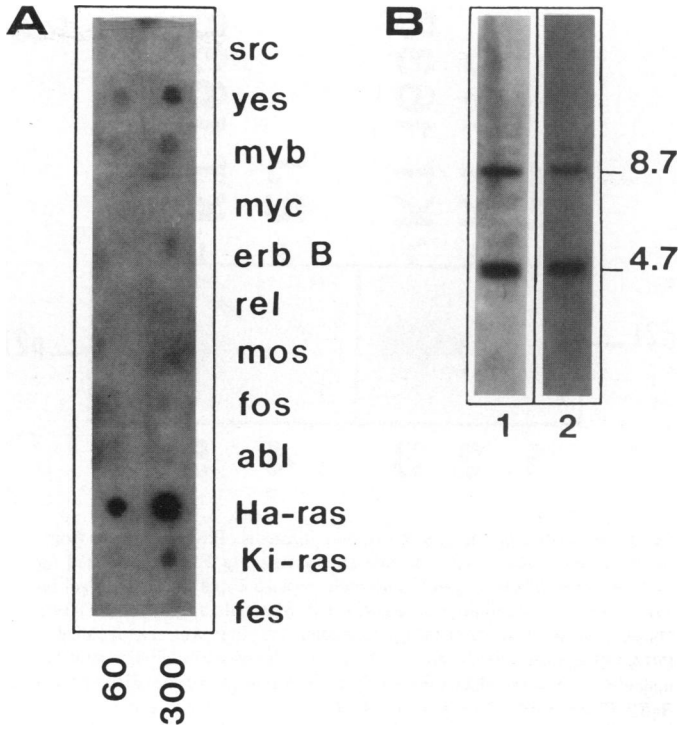

Fig. 4. Clone 34 contains cellularly derived sequences homologous to v-Ha-*ras*. **Panel A** shows the result of hybridization of probe H (described in Figure 3) to a nitrocellulose filter containing a dotted array of viral oncogene restriction fragments (a generous gift from M.Schwab). Two dilutions of each fragment, equivalent to 300 and 60 ng, are shown. **Panel B** shows an analysis of chicken DNA (Southern, 1975). Normal chicken DNA (10 μg) was digested to completion with *Eco*RI, divided in two, electrophoresed and transferred to generate duplicate nitrocellulose filter strips. One strip was annealed with a radiolabelled probe prepared from the whole 1.05-kb clone 34 *Eco*RI fragment. Under the conditions used here this probe does not detect viral sequences in genomic DNA, presumably because U5 sequences constitute <8% of the probe fragment. The other strip was annealed with the v-Ha-*ras*-specific fragment (Ellis *et al.*, 1980). Annealing protocols were identical for all the filters (40% formamide, 3 × SSC, 37°C) and the filters were washed in 1 × SSC, 0.1% SDS at 53°C.

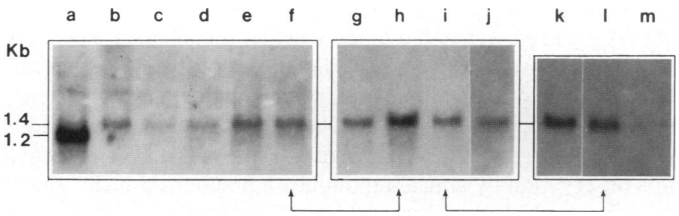

Fig. 5. Analysis of tumor RNAs with a Ha-*ras*-specific probe derived from cDNA clone 34. The figure is a composite obtained from three separate analyses of polyadenylated RNAs of species related to probe H (see text and Figure 3). Samples within each panel were electrophoresed on the same gel and hybridized and autoradiographed for the same length of time. Some RNA samples (**lanes f** and **h**, **lanes i** and **l**, indicated by arrows) are present on more than one gel, allowing the different panels to be compared. Each lane contains 5 μg of poly(A)$^+$ RNA. Ne, nephroblastoma; **lane a**, Ne1638; **lane b**, Ne890; **lane c**, Ne209; **lane d**, Ne2905, **lane e**, Ne2916; **lane f**, **lane l**, Ne2922; **lane g**, Ne196; **lane i**, **lane l**, Ne854; **lane j**, chick embryo fibroblasts; **lane k**, Ne880; **lane m**, normal kidney. Approximate sizes are shown in kilobases.

1.4-kb RNAs. Two strategies seemed feasible. The first was to obtain genomic clones of the proviruses (plus flanking sequences) that direct the synthesis of these transcripts. However, tumors 1638 and 890 both contain multiple MAV proviruses (Figure 2, lane 3; unpublished data). We therefore adopted the more direct approach of cDNA cloning. The 1.2-kb RNA of nephroblastoma 1638 was selected for this purpose.

Cloning of cDNA transcribed from the novel RNA in nephroblastoma 1638

Approximately 130 000 cDNA clones were prepared from poly(A)$^+$ RNA from nephroblastoma 1638 as described in Materials and methods. These clones were amplified and screened with a radiolabelled probe specific for the U5 domain of avian retroviral LTR. Of 10 phage identified in this way, six contained cDNA fragments apparently derived from normal proviral transcripts as they also hybridized with a MAV *env* gene probe. These phage were discarded. Of the remaining clones, the phage with the largest cDNA insert, λgt10 (clone 34), was purified for

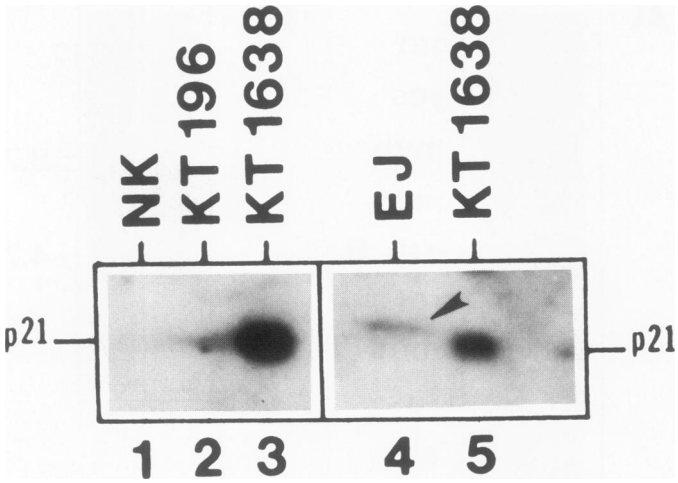

Fig. 6. Western blot analysis of c-Ha-*ras* proteins. Protein extracts from kidney tumors 196 or 1638, normal kidney tissue or EJ*ras*-transfected rat embryo fibroblasts were electrophoresed on a 15% polyacrylamide gel and transferred to nitrocellulose as described in Materials and methods. Ha-*ras*-related proteins were detected by incubating the filter with a polyclonal antiserum against denatured c-Ha-*ras* protein followed by [^{125}I]protein A. The filter was exposed to film for 36 h. The position of the EJ*ras* protein is shown by an arrow. **Track 1**: 1300 μg total protein from normal kidney. **Track 2**: 1300 μg total protein from kidney tumor 196. **Track 3**: 80 μg total protein from 1638 kidney tumor. **Track 4**: 20 μg total protein from EJ*ras*-transfected REFs. **Track 5**: 20 μg total protein from 1638 kidney tumor.

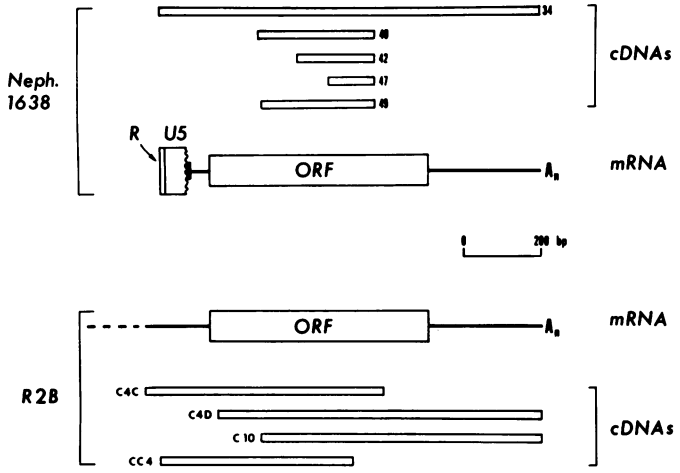

Fig. 7. Structure of cDNA clones homologous to c-Ha-*ras* mRNAs isolated from nephroblastoma 1638 and the bursal lymphoma cell line R2B. The coordinates of cDNA clones are indicated by open boxes. The structures of the R2B and 1638 Ha-*ras* mRNAs deduced from these clones are shown in the center of the figure. The truncated nature of the U5 domain in the 1638 RNA is indicated by a wavy vertical line. An adjacent block of five nucleotides in the 1638 mRNA which is not homologous to the MAV LTR or R2B Ha-*ras* sequences is represented by black shading. ORF = open reading frame. bp = base pairs. Neph. = nephroblastoma. The composite total length of the R2B cDNA (1018 nucleotides) is very similar to that of the 1638 cDNA (998 nucleotides). However, the corresponding mRNAs differ by ~200 bp (Figure 5). We conclude that we have failed to clone the 5′ extremity of the R2B mRNA; this segment is indicated by a dashed line.

further analysis. By digestion of the 1.05-kb *Eco*RI insert fragment of this phage with *Hinf*I, we generated a 240-bp fragment that did not anneal with U5 probe and was presumed to represent part of the host sequence in a chimaeric mRNA (probe H) (Figure 3, panel B). This fragment was radiolabelled and used to re-examine a nitrocellulose filter strip containing RNA from nephroblastoma 1638 (Figure 3, lane 5). Probe H detects a transcript of 1.2 kb, indicating that clone 34 is an authentic copy of the RNA detected with U5 probe. As anticipated, probe H does not anneal with genome-sized or *env* viral mRNAs.

Sequences within clone 34 share homology with the transforming gene of Harvey sarcoma virus (v-Ha-ras)

cDNA clone 34 was tested to see whether it contained sequences related to known oncogenes. Radiolabeled probe H was annealed to a 'dot-blot' containing an array of purified viral oncogene-specific restriction fragments. The v-Ha-*ras* clone BS-9 (Ellis *et al.*, 1980) gave an intense signal (Figure 4A). We extended this observation by annealing duplicate Southern transfer filters of uninfected chicken liver DNA under identical hybridization conditions with radiolabeled probe H and BS-9 (Figure 4B). The two probes detect identically sized restriction fragments in an *Eco*RI digest (8.7 and 4.7 kb). The smaller *Eco*RI fragment has been observed in a previously published analysis of chicken DNA with the v-Ha-*ras* probe (Ellis *et al.*, 1981). (Detection of the additional 8.7-kb fragment may reflect the less stringent hybridization conditions used here.) We conclude that the aberrant transcript in tumor 1638 is a hybrid MAV/c-Ha-*ras* mRNA.

Expression of c-Ha-ras mRNA in nephroblastomas and non-neoplastic tissue

We have assessed expression of c-Ha-*ras* in normal avian tissue and in other nephroblastomas (Figure 5). Polyadenylated RNAs were fractionated by electrophoresis, transferred to nitrocellulose and hybridized to radiolabeled probe H as described above. A 1.4-kb c-Ha-*ras* mRNA is detected in all samples (except 1638 RNA) including non-neoplastic kidney (lane m) and embryo fibroblasts (lane j). Densitometric analysis (not shown) indicates this mRNA is expressed as a 3- to 5-fold higher steady-state level in eight nephroblastomas than in normal kidney. The 1.2-kb MAV-*ras* mRNA in tumor 1638 is at least 25-fold more abundant than c-Ha-*ras* mRNA in normal kidney.

The 1.4-kb transcript from nephroblastoma 890 detected with U5 probe may also contain sequences from c-Ha-*ras*, as probe H detects a single 1.4-kb transcript in poly(A)$^+$ RNA (Figure 5, lane b). However, this is also the size of the putative wild-type c-Ha-*ras* mRNA. Lack of further material from tumor 890 has precluded a definitive appraisal of this possibility.

A 21-kd c-Ha-ras-related protein is overexpressed in tumor 1638

Tumor 1638 was analyzed serologically to determine the type and abundance of Ha-*ras* protein encoded by the activated allele. Crude protein extracts from tumor 1638 (Figure 6, lanes 3 and 5), normal chicken kidney tissue (lane 1), tumor 196 (lane 2) and rat embryo fibroblasts transfected with the mutant c-Ha-*ras*-1 allele derived from the human bladder carcinoma cell line EJ (Parada *et al.*, 1982) (lane 4) were electrophoresed on 15% acrylamide gels, electroblotted onto nitrocellulose and incubated with a polyclonal antisera raised against denatured human c-Ha-*ras*-1 protein (a gift from Art Levinson). Tumor 1638 contains an abundant cross-reactive 21-kd protein species which migrates more rapidly than the mutated c-Ha-*ras* protein in the EJ-transfected cells. Tumor 196 and normal kidney which contain smaller amounts of *ras* poly(A)$^+$ RNAs (Figure 4, lanes g and m) contain less Ha-*ras*-related protein. The c-Ha-*ras*-related proteins identified in normal kidney and tumor 196 extracts have the same electrophoretic mobility as the c-Ha-*ras* protein identified in nephroblastoma 1638 extracts. We conclude that tumor 1638 contains abundant levels of an apparently normal c-Ha-*ras* protein.

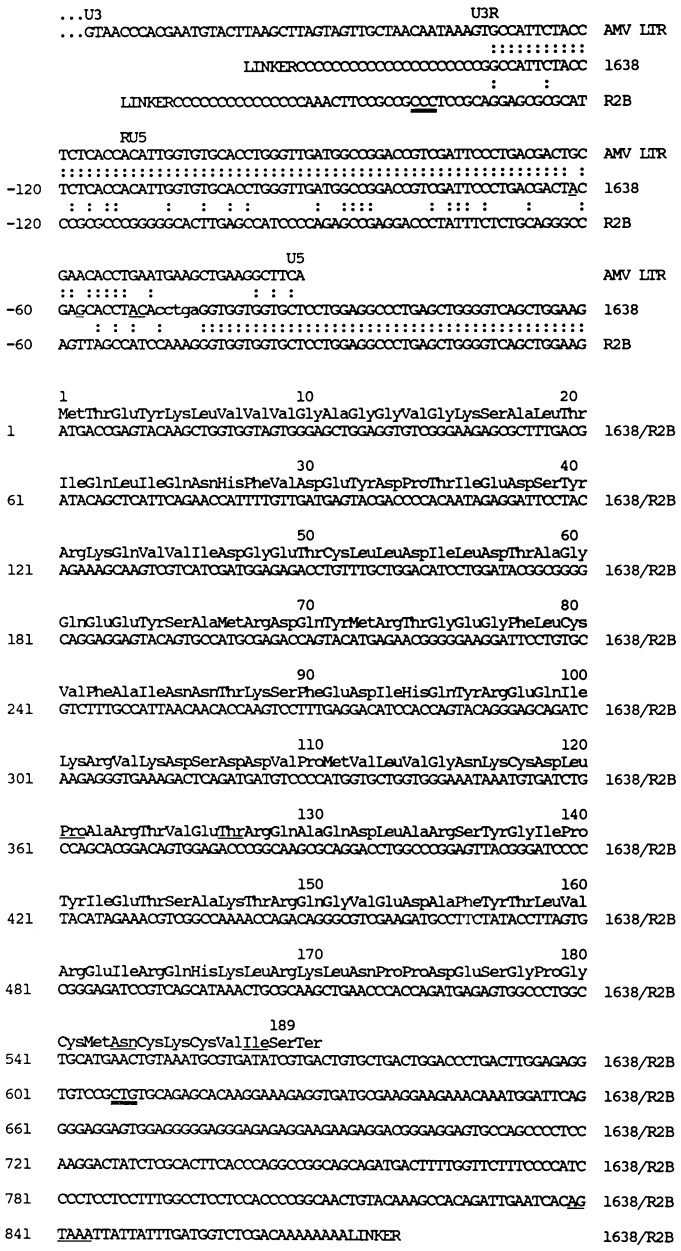

Fig. 8. The nucleotide sequence of c-Ha-*ras* cDNA clones. These sequences are numbered with respect to the Ha-*ras* open reading frame, nucleotide 1 corresponding to the A of the ATG codon. The first part of the 1638 cDNA sequence has been aligned with the sequence of an AMV LTR. The AMV sequence has been presented in the form anticipated for a provirus; hence the terminal dinucleotide (TT) of the unintegrated LTR has been omitted. Boundaries of the U3, R and U5 domains are indicated above the nucleotide sequence. Vertical bars indicate homology. Underlinings indicate deviations from the AMV sequence also observed in a MAV-2(N) sequence. Nucleotide −131 of the 1638 sequence corresponds to the putative LTR 'cap site'. Codons for amino acids which differ from the normal human Ha-*ras*-1 sequence are underlined. Five nucleotides, CCTGA, of uncertain origin in the 1638 cDNA are displayed in lower-case letters. The probable polyadenylation signal, AGTAAA, is underlined. Double-underlined nucleotides in the untranslated region are assigned tentatively as the sequencing gels yielded compressions at these positions.

This is presumably encoded by the chimaeric 1.2-kb transcript.

Nucleotide sequences of c-Ha-ras cDNAs derived from nephroblastoma 1638 and a lymphoma cell-line

The complete sequence of the nephroblastoma 1638 cDNA clone 34 was determined. To appraise whether somatic missense mutations feature in the pathogenesis of nephroblastoma 1638, we isolated and sequenced cDNA clones homologous to (presumably) normal chicken c-Ha-*ras* mRNAs. The latter cDNAs were derived from the bursal lymphoma cell line R2B (Chen *et al.*, 1983) which contains 1.4-kb Ha-*ras* mRNAs (unpublished results of D.Westaway). The coordinates of these clones are summarized in Figure 7.

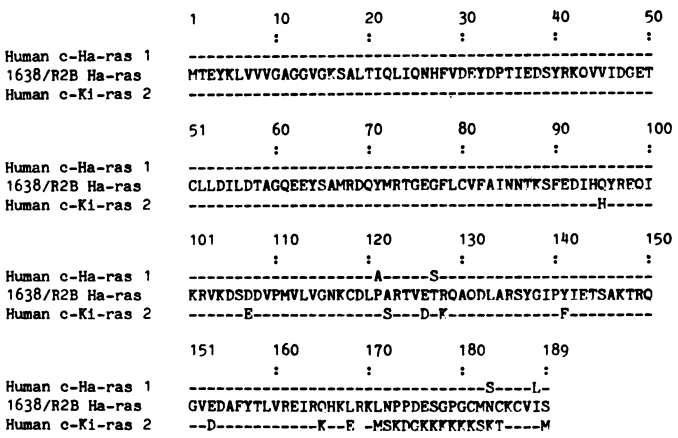

Fig. 9. Comparison of the predicted amino acid sequence of the 1638/R2B cDNAs with normal human c-Ha-*ras* and c-Ki-*ras* gene sequences. A dash indicates homology to the nephroblastoma Ha-*ras* sequence. The c-Ha-*ras*-1 sequence is from Capon *et al.* (1983a) and the c-Ki-*ras*-2 (using exon 4B) sequence is from Capon *et al.* (1983b) and McCoy *et al.* (1984). A gap of one codon has been introduced to align the carboxy terminus of the c-Ki-*ras*-2/4B sequence.

The 5′ termini of R2B- and 1638-derived cDNAs are unrelated until nucleotide −44 (Figure 8). To demonstrate the proviral origin of the 5′ terminus of the 1638 cDNA, we have aligned this area with sequences from the AMV LTR (Klempnauer *et al.*, 1982; Rushlow *et al.*, 1982). The 5′ extremity of clone 34 is defined by decameric *Eco*RI 'linker' sequences followed by a synthetic dC homopolymeric tract, the result of dG addition to the first cDNA strand by terminal deoxynucleotidyl transferase. This is flanked on the 3′ side by one residue (G) of unknown provenance (nucleotide −132). Immediately adjacent to this are sequences homologous to the R and U5 domains of an LTR avian retroviral genome. Homology extends from the putative transcription initiation site, the 'cap' nucleotide, to a position 17 nucleotides upstream of the normal 3′ boundary of the LTR. There are four nucleotides within this block of homology (nucleotides −62, −58, −52, −51) which differ from the AMV U5 sequence. These are underlined. However, these changes are also observed in the U5 sequence of a cloned MAV-2(N) LTR (K.-H.Klempnauer and D.Westaway, unpublished results). Hence the position of divergence between the AMV and the 1638 cDNA sequence has been placed between nucleotides −49 and −50. Five nucleotides of uncertain origin occur before homology with the R2B cDNA commences. These nucleotides occur at positions −46 to −50 in the 1638 sequence, and are shown in lower-case letters.

Why does the 1638 cDNA contain a truncated U5 sequence? The sequence around the breakpoint of homology between the AMV LTR and the cDNA is (in the intact LTR) unrelated to the sequence of a consensus splice donor site, AG/GTRAG (Sharp, 1981). Therefore, the 'missing' U5 nucleotides of clone 34 cannot readily be attributed to a splicing event. Nor can the missing nucleotides be ascribed to a deletion generated in m13 subcloning as this region of the clone has also been sequenced by the chemical degradation method, using a plasmid subclone. Two possibilities remain. Either the provirus linked to c-Ha-*ras*

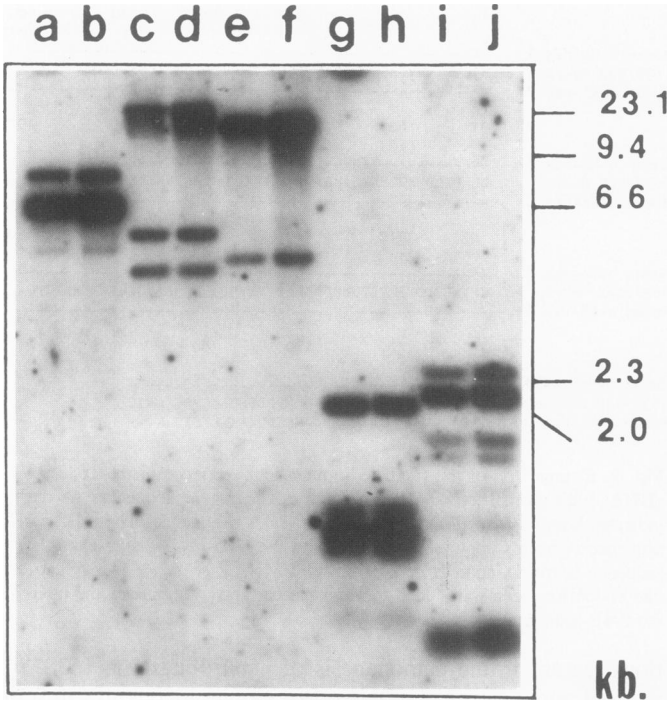

Fig. 10. Seeking a disrupted c-Ha-*ras* allele in nephroblastoma 1638 DNA. Digests of non-neoplastic (heart) or nephroblastoma DNA from bird 1638 were fractionated on 1% agarose gel and transferred to nitrocellulose filters, and hybridized with the Ha-*ras*-specific clone C4C described in Figure 7. **Lanes a, c, e, g, i** = non-neoplastic control (heart) DNA from bird 1638. **Lanes b, d, f, h, j** = nephroblastoma 1638 DNA. **Lanes a** and **b** = *Hind*III; **lanes c** and **d** = *Hpa*I, **lanes e** and **f** = *Kpn*I; **lanes g** and **h** = *Pst*I; **lanes i** and **j** = *Pvu*II.

is defective, or a small deletion occurred during cDNA cloning.

Clone 34 and the R2B *ras* mRNA are almost perfectly concordant from nucleotide −44 to 866. In the R2B *ras* mRNA sequence this area includes 44 nucleotides of a putative 5′-untranslated region followed by an 189-codon open reading frame, and a 299-nucleotide 3′-untranslated region. The clone 34 coding region differs from the R2B sequence at one position; the R2B sequence TCCCCA at nucleotides 326−331 is replaced by TCCCA. The sequence of clone 34 would predict termination at a TAG triplet at nucleotides 425−427, resulting in an ~17-kd protein. However, Western blot analysis demonstrates only a 21-kd *ras* protein in nephroblastoma 1638 (Figure 6). This single nucleotide discrepancy probably represents a deletion generated during reverse transcription of the chimaeric transcript. To verify this interpretation, additional cDNA clones were generated from nephroblastoma 1638 RNA (Figure 7). All four analyzed contain the sequence TCCCCA. We conclude that the R2B and chimaeric Ha-*ras* mRNAs encode identical 21-kd Ha-*ras* proteins.

Predicted amino acid sequence of the chicken c-Ha-ras protein

The predicted amino acid sequence deduced from the open reading frames of the R2B and 1638 cDNAs has been aligned with amino acid sequences from germline alleles of human c-Ha-*ras*-1 and c-Ki-*ras*-2 (using exon 4B) (Figure 9). Only four codons differ between the chicken and human c-Ha-*ras*-1 sequence; these do not include codon variants at positions 12, 13 or 61 which are characteristic of transforming *ras* alleles isolated via transfection of NIH/3T3 cells (Tabin *et al.*, 1982; Reddy *et al.*, 1982; Taparowsky *et al.*, 1982; Capon *et al.*, 1983a, 1983b; Sukumar *et al.*, 1983; Yuasa *et al.*, 1983; Fasano *et al.*, 1984; Bos *et al.*, 1985). Two of these codons (183, 188) lie at the carboxy terminus, an area that is poorly conserved between the Ha-*ras*-related genes of different species (Powers *et al.*, 1984; Neuman-Silberberg *et al.*, 1984). The remaining two codons at 121 and 127 are positions at which human c-Ha-*ras*-1 and c-Ki-*ras*-2 differ. These results provide a further illustration of the evolutionary conservation of *ras* genes.

Attempts to define a c-Ha-ras-linked proviral insertion

The anticipated template for the chimaeric transcript in nephroblastoma 1638 is a provirally disrupted chicken c-Ha-*ras* gene. We have sought evidence for such a structure by the analysis of genomic DNA. High mol. wt. DNAs from nephroblastoma 1638 and non-neoplastic control tissue were digested to completion with a variety of restriction endonucleases, electrophoresed on agarose gels and transferred to a nitrocellulose filter. This filter was hybridized with radiolabeled copies of the R2B cDNA clone C4C. (This clone contains the most complete copy of the mRNA's 5′-untranslated region.)

In none of the pair-wise gel loadings (Figure 10, lanes a−j) can we detect a *ras*-related fragment present in the tumor sample but not present in the control tissue. Similar negative results have been obtained with other nephroblastoma DNAs (not shown). The use of a variety of restriction enzymes in this experiment makes it unlikely that a trivial explanation (e.g., comigration or poor transfer to nitrocellulose of excessively large or small restriction fragments) underlies the failure to detect a somatically rearranged c-Ha-*ras* allele.

Discussion

Seeking a target for insertional mutations in avian nephroblastomas

MAV-induced nephroblastomas appear clonal in tests of integrated proviruses (Figure 2), a phenomenon shared by many well characterized instances of insertional mutation of oncogenes (Fung *et al.*, 1981, 1982; Neel *et al.*, 1981; Payne *et al.*, 1981; Nusee and Varmus, 1982; Peters *et al.*, 1983; Tsichlis *et al.*, 1983). Clonality has also been observed in avian nephroblastomas induced by ring-neck pheasant virus (RPV) (Simon *et al.*, 1984) and MAV 2-(0) (Boni-Schetzler *et al.*, 1985). Our strategies to uncover specific insertional targets via the examination of genomic DNA using probes for known oncogenes or the 'transposon tagging' procedure have failed. However, a strategy focusing on altered patterns of transcription has met with some success.

Two nephroblastomas contain small, novel transcripts which anneal with viral U5 LTR probe but not with a probe that includes the proviral polyadenylation signal. Precedents from the proviral activation of c-*myc* and c-*erb*-B suggested that these RNAs may be derived from LTR-initiated transcription into cellular sequences. We validated this hypothesis by obtaining cDNA clones homologous to one of these transcripts. cDNA nucleotide sequences reveal that the 1.2-kb RNA in nephroblastoma 1638 is initiated within a MAV LTR situated upstream of a c-Ha-*ras* gene.

Proviral activation of an avian ras gene in nephroblastoma 1638

We estimate that the chimaeric Ha-*ras* mRNA in nephroblastoma 1638 is 25-fold more abundant than its counterpart in normal renal tissue (Figure 5). This is paralleled by an increase in expression of a 21-kd Ha-*ras* protein (Figure 6). Most other characterized instances of *ras* gene activations *in vivo* appear to involve missense mutations at codons 12, 13 and 61. Do such

point mutations feature in the pathogenesis of nephroblastoma 1638? The cDNA sequence derived from the bipartite transcript encodes amino acids at codons 12, 13 and 61 that are characteristic of wild-type Ha-*ras* genes. Moreover, this predicted amino acid sequence is completely congruent with one derived from a Ha-*ras* cDNA isolated from the bursal lymphoma cell line R2B. We conclude that the activated c-Ha-*ras* allele in nephroblastoma 1638 has not sustained missense mutations in the Ha-*ras* coding sequence.

Is there evidence to suggest that augmented expression of a Ha-*ras* gene bearing normal codons at 12, 13 and 61 has an impact on the transformed phenotype? Several examples exist. Normal rat and human c-Ha-*ras* genes activated by murine leukemia virus LTRs rendered NIH/3T3 cells tumorigenic *in vivo* (Chang *et al.*, 1982). Similar results are obtained when c-Ha-*ras* gene dosage is increased by transfection (Pulciani *et al.*, 1985). In another instance, a transcriptionally activated normal human c-Ha-*ras* gene was capable of rescuing early passage rodent cells from senescence and also of converting 'immortalized' cells to anchorage independence (Spandidos and Wilkie, 1984). Amplified Ki-*ras* alleles, encoding wild-type p21 proteins have been identified in the murine adrenocortical cell line Y-1 (Schwab *et al.*, 1983; George *et al.*, 1985). A Ki-*ras* amplification was also observed in a human lung carcinoma (Pulciani *et al.*, 1985).

We have yet to detect the Ha-*ras*-linked MAV provirus in nephroblastoma 1638. Five different restriction endonucleases were used for the analysis presented in Figure 10. With all of these, DNA from nephroblastoma 1638 yields the same pattern of hybridizing fragments as control DNA. A trivial explanation is that cells harboring the insertional mutations are underrepresented in the tumor mass (i.e. insertional activation is a late event) such that normal c-Ha-*ras* alleles predominate. Three observations make this unlikely. First, the autoradiograms presented in Figure 10 were deliberately overexposed to reveal such submolar fragments. Second, proviral junction fragments are seen in the same DNA when hybridized with an LTR-specific probe (Figure 2, lane 3). Third, four independent biopsies of nephroblastoma 1638 used to prepare poly(A)$^+$ RNA (two shown here, Figure 3, lane 5 and Figure 5, lane a) contain the 1.2-kb chimaeric transcript. One more likely explanation is that the activating provirus is extensively deleted such that restriction fragments derived from the disrupted c-Ha-*ras* allele cannot be distinguished from their wild-type homologues. By analogy with activated c-*myc* alleles, the minimum structure for an activating provirus may be a single LTR (Westaway *et al.*, 1984). A *ras* allele bearing such an insertion will be revealed by using restriction endonucleases which cleave within the MAV LTR. Two such restriction enzymes, *Hind*III and *Hpa*I (Klempnauer *et al.*, 1982; Rushlow *et al.*, 1982) are included in our analysis (Figure 10, lanes a – d). Another possibility is that the activating provirus lies in an area of the chromosome that is not detected by the radiolabeled cDNA clone C4C. For example, a proviral insertion adjacent to a distant, normally cryptic, splice donor site may go unnoticed. This model could also account for the five nucleotides, CCTGA, of unknown provenance within the 1638 RNA.

Activated ras genes and nephroblastomas

This is the first report of an activated c-Ha-*ras* gene in a nephroblastoma. Nine other MAV-induced nephroblastomas examined by us do not appear to harbor transcriptionally activated Ha-*ras* genes (Figure 5). Thus tumor 1638 may be unique and does not define an oncogene which is crucial for nephroblastomagenesis. It is conceivable that the activation of another type of *ras* gene is the common genetic event in MAV-induced kidney tumors. However, an earlier attempt to define a chromosomal domain which was disrupted in independent tumors failed (Nusse *et al.*, 1984a; D.Westaway, unpublished observations).

The c-Ha-*ras* gene is also unlikely to figure in the genesis of the human nephroblastoma, Wilms' tumor. This tumor appears to result from progression to homozygosity for recessive chromosomal lesions (Knudson and Strong, 1972; Riccardi *et al.*, 1978; Francke *et al.*, 1979; Yunis and Ramsay, 1980; Huerre *et al.*, 1983; Fearon *et al.*, 1984; Koufos *et al.*, 1984; Orkin *et al.*, 1984), an observation that is incompatible with the dominant nature of activated c-Ha-*ras* alleles.

An alternative strategy for the identification of insertional lesions in tumors

Crucial insertional mutations in tumor DNAs may be identified using known oncogene probes or cellular restriction fragments which abut novel proviral insertions. However, these strategies can occasionally yield false negative results, e.g. if the insertional 'target' gene is very large or does not share homology with currently known oncogenes.

We have used an alternative strategy to identify the chicken c-Ha-*ras* gene as a substrate for insertional activation. This approach depends upon the identification of candidate bipartite proviral host transcripts in tumor RNA. Such species are encountered at a reasonable frequency in some of the well-characterized instances of insertional mutation (Neel *et al.*, 1981, 1982; Payne *et al.*, 1981, 1982; Fung *et al.*, 1983; Nusse *et al.*, 1984b; van Ooyen and Nusse, 1984; Muller and Muller, 1984; Varmus *et al.*, 1981; Wolf and Rotter, 1984). cDNA clones prepared from such tumor RNA are screened with a probe specific for the LTR. Recombinants derived from the normal proviral transcripts are discarded. Barring cloning artefacts, the remaining recombinants will be derived from chimaeric RNAs. This approach can be used when the tumor harbors multiple proviruses, e.g. nephroblastoma 1638 contains many MAV proviruses integrated in both a clonal and non-clonal fashion (Figure 2, lane 3), but contains only three abundant provirally related RNA species, the bipartite MAV/c-Ha-*ras* mRNA and the usual genome-sized and *env* mRNAs (Figure 3, lane 3).

Materials and methods

Source of tumors

Three isolates of MAV were used for these studies. The first was MAV-2(N) originally isolated by Ogura *et al.* (1974) and provided by Dr Ralph Smith. This isolate was subjected to four cycles of plaque purification as described by Moscovici *et al.* (1976). This yielded a virus stock with a titre of 1×10^7 p.f.u./ml which was injected into the metatarsus vein of day-old chicks. A second isolate was the MAV-1 stock described by Smith and Moscovici (1969). The route of administration was as above. The third isolate was prepared by infection of secondary macrophages with a mixture of MAV-1 and MAV-2 (Moscovici and Vogt, 1968). The next day, 10^7 trypsinized macrophages were fused with inactivated Sendai virus. Seven- and 14-day supernatants of the cultures were harvested and injected into 13-day-old embryos via the chorioallantoic vein. Chicken flocks used were SPAFAS (MAV-1 and MAV-2) and Hyline or Line 6 [MAV-2(N), MAV-1]. Host susceptibilities ranged from 70% (Hyline) to 100% (line 6). Average age at sacrifice was 75 days.

Isolation of tissue RNA

Biopsies from tumors and normal organs (~1 g) were partially thawed, sliced with a razor blade, suspended in 10 ml of 4 M guanidinium isothiocyanate, 50 mM Tris-HCl pH 7.6, 10 mM EDTA, 1% sarkosyl, 1% 2-mercaptoethanol, and disrupted in a polytron mixer. The homogenate was then extracted with phenol/chloroform/isoamyl alcohol (24:24:1) precipitated wtih alcohol, treated wtih Proteinase K and re-extracted as described by Feramisco *et al.* (1982). Nucleic acids were recovered by ethanol precipitation and polyadenylated RNA selected by one cycle of chromatography on oligo(dT)-cellulose (Aviv and Leder, 1972).

Isolation of high mol. wt. DNA

Approximately 1 g of tissue was partially thawed, minced with a razor blade and resuspended in 10 ml of a 10 mM Tris-HCl pH 8.0, 25 mM EDTA, 200 μg/ml proteinase K, and 25% v/v glycerol. The tissue was disrupted by several strokes of a motor-driven Dounce homogenizer with a Teflon head. Fragments of connective tissue were removed and SDS was added to a final concentration of 1%. The solution was incubated at 53°C for 3 h, and extracted twice with an equal volume of phenol:chloroform and twice with chloroform:isoamyl alcohol (24:1). DNA was recovered by spooling after the additon of 2 volumes of ice-cold ethanol. The dry DNA pellet was dissolved in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and incubated with RNase A (100 μg/ml) for 60 min at 37°C, followed by Proteinase K (200 mg/ml, 60 min, 37°C). The solution was subjected to the extraction procedures detailed above, ethanol precipitated and resuspended in TE to a concentration of 250 μg/ml.

Molecular cloning

Nephroblastoma 1638 RNA was isolated as described above and subjected to a further cycle of oligo(dT)-chromatography. Synthesis of double-stranded cDNA followed the protocol of T.St.John (personal communication). Briefly, cDNA was synthesized on oligo(dT)-primed RNA under standard conditions. The DNA strands were tailed with dG residues and treated with RNase A. Second strand synthesis by DNA polymerase I was primed with oligo(dC). The double-stranded cDNA was methylated with *Eco*RI methylase and ligated to synthetic *Eco* linkers. After digestion with *Eco*RI, double-stranded cDNAs were size selected on a 2% agarose gel electrophoresed in Tris-acetate buffer. cDNA fragments >0.6 kb were electroeluted, phenol-extracted, desalted, lyophilized and ligated to *Eco*RI-digested λ_{gt10} (Huynh *et al.*, 1985). The vector DNA exhibited a basal clear plaque frequency of $0.23 + 0.03 \times 10^{-2}$ (N=4) after ligation and *in vitro* packaging. Ligation of cDNAs to vector were carried out at four arbitrarily chosen ratios yielding clear plaque frequencies between 10^{-2} and 0.3. Products of these reactions were pooled and parental phage were counterselected by amplification on the Hfl $r_k^- m_k^+$ strain NM514 (Murray, 1983, a gift from Peter Little). The amplified progeny phage were replated on NM514 and screened by plaque hybridization (Benton and Davis, 1977) with a radiolabelled U5-specific hybridization probe. Phage which scored positive on duplicated plaque lifts were rescreened with the 'env' hybridization probe shown in Figure 1. Phage which annealed with U5, but not with 'env' probe were plaque purified and their DNA analyzed (Cameron *et al.*, 1977).

A second batch of cDNA was generated from 1638 RNA via an abbreviated procedure. First strand synthesis was primed via a mixture of oligo(dT) and a synthetic oligonucleotide dTTCTATGTAGGGGATCCCGTAACT complementary to codons 136–143. *Eco*RI linkers were removed from the double-stranded cDNA by chromatography over an ion exchange column ('NACS', Bethesda Research Laboratories). The resultant cDNA was cloned directly into λ_{gt10} as described above, and phage screened without amplification using a probe excised from clone 34. The probe extended from the *Cla*I site (nucleotide 138) to the *Nco*I site (nucleotide 330; Figure 8).

c-Ha-*ras* clones were isolated from a λgt10 cDNA library (a gift from Ling-Chun Chen) prepared from RNA from the bursal lymphoma cell line R2B. The library was screened with a radiolabeled *Pvu*II-*Eco*RI fragment excised from the 1638-derived cDNA clone 34. Four overlapping clones were isolated and are detailed in Figure 7.

DNA sequencing

The *Eco*RI insert of clone 34 was subcloned in the plasmid pUC8 (Vieira and Messing, 1982) by standard procedures. This subclone was digested with *Eco*RI and the 1.05-kb insert isolated by gel electrophoresis. This fragment was rendered blunt-ended by treatment with the Klenow fragment of DNA polymerase I (Drouin, 1980), and digested with *Alu*I, *Hae*III or *Rsa*I. Fragments from these digests were cloned into the *Sma*I site of the m13 vector mp18 (Norrander *et al.*, 1983). Sequencing was performed as described by Biggin *et al.* (1983). Additionally, clone 34 was sequenced via the chemical degradation method after radiolabeling at *Eco*RI, *Nco*I and *Cla*I restriction sites (Maxam and Gilbert, 1980). Molecular clones derived from the second batch of 1638 cDNA were subcloned directly into mp18 cleaved at the *Eco*RI site.

The *Eco*RI inserts from all four R2B-derived gt10 clones were subcloned directly into the *Eco*RI site of the M13 vector mp18. In addition, the *Eco*RI insert from the clone C4C was subcloned into the plasmid pUC8. This pUC8 subclone was used as a source of smaller fragments (*Eco*RI-*Pvu*II, *Nco*I-*Eco*RV) which were blunt ended with *Escherichia coli* DNA polymerase I and ligated into the *Sma*I site of M13 mp18 or *Eco*RI-*Cla*I which was ligated to the *Eco*RI-*Acc*I-digested M13 mp18. Finally, the replicative form of the complete M13-C4C subclone was purified, digested with *Xba*I and *Nco*I, blunt-ended and religated to form an additional substrate for sequencing. All c-Ha-*ras* M13 subclones were sequenced (in both orientations) using the dideoxy procedure. With the exception of 60 nucleotides in the R2B 5′-untranslated region, all sequences were determined on both strands.

Analysis of tumor RNAs

Polyadenylated RNA (5 μg) was ethanol precipitated and electrophoresed on 0.8% agarose-formaldehyde gels and transferred to nitrocellulose filters after the method of Thomas (1980), as described by Nusse and Varmus (1982).

Analysis of cellular DNA with restriction endonucleases

These techniques are described by Payne *et al.* (1981).

Source and preparation of molecular hybridization probes

The locations of proviral hybridization probe fragments are shown in Figures 1 and 2. Fragments were recovered as described by Westaway *et al.* (1984) and were radiolabeled by random priming as described by Payne *et al.* (1981).

Western blot analysis of Ha-ras proteins

Small biopsies of frozen kidney tumors 196 or 1638 and normal kidney tissue were pulverized in a Dounce homogenizer containing 20 mM Tris, pH 8.0, 1 mM dithiothreitol (DTT), 1 mM EDTA and 1% aprotinin. A washed cell pellet from one 9-cm dish of EJ*ras* transformed rat embryo fibroblasts was processed in the same manner. The homogenates were centrifuged for 5 min in a microfuge and a standard Bio-Rad protein determination was made on the supernatant. Aliquots of the supernatants containing 20 μg (tracks 1 and 2), 1300 μg (tracks 3 and 4) or 80 μg (track 5) of total protein were mixed with an equal volume of 2 × sample buffer, boiled for 1 h and loaded onto a 2-mm thick 15% acrylamide gel. Pre-stained high mol. wt. protein markers (BRL) were electrophoresed in parallel.

After electrophoresis, the gel was transferred directly to nitrocellulose using a Bio-Rad trans-blot apparatus. After transfer the nitrocellulose filter was washed for ~1 h with several changes of phosphate-buffered saline (PBS) and PBS plus 2% non-fat dry milk and 0.1% NP40 (PMN). The filter was then incubated for 3 h with 5 ml of PMN solution containing 50 μl polyclonal anti-p21 serum. After washing for 1 h with several changes of PMN, the filter was again incubated with 6 ml of the same containing 1.5 mCi [^{125}I]protein A (Amersham, 30 mCi/mg) for 1 h. All manipulations were performed at room temperature. After a final wash as above, the filter was exposed to film.

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