

DIFFERENT MECHANISMS OF RAS PROTO-ONCOGENE OVEREXPRESSION
DETECTED BY A SIB-SELECTION TUMORIGENESIS ASSAY

by
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ABSTRACT

Title of Dissertation: Different Mechanisms of ras Proto-Oncogene Overexpression Detected by a Sib-Selection Tumorigenesis Assay

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Human insulinoma and renal cell carcinoma DNAs were analysed by a sib-selection tumorigenesis assay to detect the presence of dominant acting oncogenes. Although no novel oncogenes were detected in the five tumor DNAs tested, the increased sensitivity of the tumorigenesis assay allowed detection of overexpressed ras proto-oncogenes activated during transfection. Tumorigenic overexpression of ras proteins occurred by two different mechanisms: gene amplification, and increased translation efficiency of a fusion protein.

Thirty to fifty fold amplification of a human N-ras gene was detected in primary and secondary mouse tumors. All tumors containing amplified copies of N-ras overexpressed p21 and the cloned gene had no coding sequence mutations. Since DNA from the original human tumor and several metastases did not show N-ras gene amplification, it was concluded that amplification had occurred during the primary tumorigenesis assay.

In another series of tumors co-integration of the mouse c-H-ras gene during the secondary transfection resulted in tumorigenic overexpression of ras protein without elevation of mRNA levels. Three c-H-ras cDNA clones from secondary tumor RNA contained no coding sequence mutations, but were divergent at the 3' end. Polymerase chain reaction amplification of RNA showed novel alternative splicing at intron E of mouse c-H-ras mRNA in both tumors and untransfected cells.

An upstream ATG was identified that potentially initiates translation of an open reading frame (ORF) overlapping the H-ras p21 translation initiation site. A single base deletion within exon -1 of the cDNA clones placed this upstream ATG in frame with the ras coding sequence, creating a potential fusion protein. Translation of this mRNA in rabbit

reticulocyte extracts and Xenopus oocytes showed exclusive production of a p23 ras fusion protein. When the upstream ATG was deleted, only p21 ras was translated in both systems. Based on these results it is proposed that in vivo recognition of the upstream ATG and translation of the ORF overlapping the p21 start site might serve to modulate the translation of p21. The single base deletion and resultant ras fusion protein may constitute a novel mechanism of ras overexpression by circumventing this translational regulation.

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INTRODUCTION

Tumorigenesis is a multistep process involving several genetic changes and presumably the activation of more than one oncogene. Activated oncogenes have been isolated from many tumors and tumor cell lines by induction of focus formation in NIH 3T3 cells (reviewed in Cooper 1983, Bishop 1987). The first human oncogene isolated by focus formation in NIH 3T3 cells, c-H-ras1, was found to be the cellular homolog of the retroviral v-H-ras transforming gene of Ha-MSV (Der et al 1982, Santos et al 1982, Parada et al 1982, Goldfarb et al 1982), activated by a single point mutation at codon 12 (Pulciani et al 1982b, Shih et al 1982, Tabin et al 1982, Taparowsky et al 1982, Capon et al 1983). The oncogenes most often detected by the classic focus formation assay (Shih et al 1979) are members of the ras family of GTP binding proteins with activating point mutations. Since ras genes may be activated in up to 40 % of human tumors, the three members of this gene family, H-, K-, and N-ras, have been extensively studied (reviewed in Barbacid 1987). Ras genes can become tumorigenic either by point mutations in two regions involved with GTP binding and hydrolysis, or by overexpression of the normal gene product (reviewed in Sistonen and Alitalo 1986). Even though the ras literature is vast, the function(s) of the mammalian ras genes remain largely speculative, and studies of the regulation of ras gene expression are incomplete. The ras genes have unusual promoters high in G + C content like in some of the housekeeping genes (Ishii et al 1985). Recently, Cohen et al (1989) have reported a mechanism of alternative splicing in human c-H-ras that post-transcriptionally down-regulates gene expression by defective splicing.

Tumorigenesis assays have been developed to detect oncogenes that produce less profound morphological changes than those required for focus formation in transfected NIH 3T3 cells (Blair et al 1982, Keath et al 1984, Fasano et al 1984b, Bos et al 1985, Hirai et al 1987). These assays, however, were designed to detect the activation of only the most

dominant acting oncogene in the donor tumor preparation. Again, mutant ras oncogenes produce tumors at a much faster rate than myc oncogenes or overexpressed ras proto-oncogenes (Keath et al 1984, Fasano et al 1984b). Therefore, their presence would obscure the activity of any other oncogene also present in the donor DNA. The tumorigenesis assay was modified to increase the chance of detecting both strongly and weakly transforming oncogenes from the original tumor DNA. Cells co-transfected with a selectable marker were sib-selected by separating colonies of transfectants into pools that potentially represent different parts of the donor cell genome for tumorigenesis testing. Using this method, two human tumor types, pancreatic insulinoma and renal cell carcinoma, were screened for the presence of detectable transforming genes. The nude mouse tumor DNAs were analyzed for activated oncogenes, and the mechanisms by which ras proteins can become overexpressed in nude mouse tumors were investigated.

ONCOGENES: HISTORY AND PRESPECTIVE

A. RNA Tumor Viruses

Although RNA tumor viruses were first detected over eighty years ago, it wasn't until early in this decade that many of the connections between viral oncogenes and cellular proto-oncogenes were discovered. Ellerman and Bang (1908) first showed that chicken leukemia was transmissible through cell free extracts. This finding was generally disregarded at the time since leukemia was not yet classified as a form of cancer. In 1910, Peyton Rous set the cornerstone of the field of cancer virology by demonstrating the transmission of chicken sarcoma through cell free extracts of tumor tissue (Rous 1910). In the era from 1910 through the 1930's avian sarcoma virus (ASV) was discovered and shown to fulfill Koch's postulates. In rapid succession, many other viruses were isolated from tumors of different species including: rabbit papillomas (Shope 1933), mouse mammary tumors (Bittner 1936), and frog kidney carcinoma (Lucke' 1938). Studies on the papilloma virus of wild rabbits and mouse mammary tumor virus (MMTV) revealed several limitations to the application of Koch's postulates in evaluating the etiology of tumors. During the experimental transmission of papilloma virus to domestic rabbits, Shope demonstrated that after inoculation, the virus entered into an eclipse phase and was no longer detectable as an infectious agent in the tumor that it caused (Shope 1933). This effect was host dependent since the virus did not eclipse in wild rabbit tumors. Bittner's studies on the etiology of MMTV induced mammary tumors revealed that there was a long latency period between infection with the virus through the mother's milk and the appearance of mammary tumors in adulthood (Bittner 1936). He also found that tumor formation depended on both host genetic susceptibility factors and the interaction of hormones with the 'milk influence', MMTV, since only females developed tumors (Bittner 1952).

As different strains of inbred mice were developed and studied, it was noted that some strains showed a high incidence of developing certain cancers while other strains remained essentially cancer resistant. Bittner had demonstrated that the high incidence of mammary tumors in the C3H inbred mouse strain was due to virus infection. Gross was the first to show that the high incidence of leukemia in the AK mouse strain was also due to a virus. After many years of unsuccessful attempts by himself and others to "infect" weanling or older mice with leukemia, Gross inoculated newborn mice with a supernatant extract of leukemic tissue and succeeded at inducing leukemia in a mouse strain otherwise resistant to the disease (Gross 1950). In further studies, he showed that murine leukemia virus (MuLV) was vertically passaged from one generation to the next (Gross 1951), a relatively new concept at the time for the spread of infectious agents. Due to the crude methods used for virus isolation from animal tissues, many laboratories could not reproduce Gross' results until he developed a high titer virus stock, passage "A" (Gross 1957).

The increased use of tissue culture in the 1950's and the development of new techniques for the cultivation of viruses in vitro aided the propagation and detection of RNA tumor viruses. In 1952 Dulbecco adapted the bacteriophage plaque assay to quantitate animal viruses in cell culture (Dulbecco 1952). Manaker and Groupe' (1956) demonstrated that infection of chicken embryo cell cultures with ASV induced the formation of foci of transformed cells. In 1958, Temin improved the assay by using a sparse instead of crowded monolayer for infection (Temin and Rubin 1958). This quantitative focus formation assay became a model for the development of assays for other RNA and DNA tumor viruses.

Even though the in vitro focus formation assay provided a quick screening method for the presence of transformation competent viruses, no conditional lethal mutants of RSV were isolated until 1969. Toyoshima reasoned that the restrictive temperature for an avian virus should be high since the normal body temperature of chickens is 42°C. He was the first to isolate temperature sensitive and transformation defective RSV mutants (Toyoshima and Vogt 1969). The availability of these mutants led to the localization of the src gene within the

RSV genome (Duesberg and Vogt 1970, Martin 1970). The first evidence that a viral oncogene was derived from the host cell genome was obtained by hybridization of the src gene of RSV with avian genomic DNA (Stehelin et al 1976). The src gene product was identified as a 60 kd phosphoprotein not essential for viral function (Brugge and Erikson 1977). RSV is the only known acutely transforming retrovirus that contains all essential genes for replication along with a transduced oncogene. All other transforming retroviruses are replication defective since the oncogene they carry has replaced part of the essential replication genes, as discussed below.

Most of the rapidly transforming murine sarcoma viruses (MSVs) have been discovered during the passage of MuLV in animals. The first acutely transforming MSV was isolated by Harvey in 1964. She serially passaged Moloney murine leukemia virus (Mo-MuLV) in rats and then inoculated filtered plasma from leukemic rats into newborn BALB/c mice. Surprisingly, the mice rapidly developed sarcomas at the site of infection, instead of the expected leukemia (Harvey 1964). Moloney also isolated a sarcoma virus after passage of his MuLV strain in newborn BALB/c mice, Moloney-MSV (1966). Kirsten and Mayer isolated another murine sarcoma virus from their strain of MuLV which normally induced erythroblastosis in rats (Kirsten and Mayer 1967). BALB-MSV was isolated from an old mouse that developed a 'spontaneous' chloroleukemia during a study to determine the incidence of tumorigenesis in BALB/c mice (Peters 1974). Another MSV, Ra-MSV, was isolated more recently from a c-type retrovirus producing rat embryo cell line that spontaneously transformed after a long period of culture in vitro (Rasheed et al 1978).

Soon after the isolation of Moloney MSV, an in vitro focus formation assay, similar to the assay for ASV, was developed using BALB/c mouse embryo cells (Hartley and Rowe 1966). Another cell line, NIH 3T3, was established in the late 1960's to assay MSV focus formation (Jainchill et al 1969). It was originally thought that focus formation by the replication defective MSVs required help by replication competent MuLV. In 1970, it was demonstrated that the defective MSV's could induce focus formation alone in BALB/c

established mouse cell lines (Aaronson et al 1970). The helper MuLV was only needed for MSV replication and in concert they produced more rapid focus formation by infection of neighboring cells.

In the 1960's, two hypotheses to explain the origin of cancer and cancer causing viruses were proposed. Temin proposed the provirus hypothesis, that oncogenic RNA viruses arose spontaneously during DNA to RNA to DNA information transfer (Temin 1964). This idea never became widely accepted until the discovery of the retroviral enzyme that could transcribe RNA into DNA, reverse transcriptase, provided a possible mechanism for the process (Temin and Mizutani 1970, Baltimore 1970). Huebner and Todaro proposed the "oncogene hypothesis" that carcinogens, radiation or host genetic factors can cause tumors by inducing expression of retroviral genes resident in the genome of most, if not all animals (Huebner and Todaro 1969, Todaro and Huebner 1972). It was subsequently shown that the oncogenes that become activated are host genes not endogenous viral genes (src: Spector et al 1975, Stehelin et al 1976, ras: Scolnick and Parks 1974, mos: Scolnick et al 1975, Oskarsson et al 1980). Retroviral replication includes reverse transcription of the viral RNA genome into DNA and integration into the host genome (reviewed in Hughes 1982). Rare recombination events occur in which host genes are fortuitously picked up by the retrovirus, usually at the expense of one of the viral genes necessary for replication. Therefore during the passage of murine leukemia viruses in rodents, as described above, several acutely transforming MSVs were generated by the acquisition of activated oncogenes from the host during illegitimate recombination events. Both Ki-MSV and Ha-MSV were shown to contain acquired rat sequences (Scolnick et al. 1973, Scolnick and Parks 1974), and a normal cellular protein could be detected with anti-viral antibodies in uninfected cell extracts (Langbeheim et al 1980). The proto-oncogene and oncogenic forms of these genes were later identified as members of a gene family (Ellis et al 1981) termed ras from its original isolation from a rat sarcoma. These two MSVs also

contain sequences from rat (30S) endogenous retroviruses and therefore must have originated from complex recombinational events (Chien et al 1979).

Retroviral oncogenes, termed 'v-onc' genes, are transduced, intron-less, usually mutant copies of cellular oncogenes, termed 'c-onc' genes (Bister and Jansen 1986). With the exception of Mo-MSV, which contains the v-mos gene, the replication defective MSVs were generated by transduction of host derived ras genes (see Lacal and Tronick 1988 for review). BALB-MSV contains acquired mouse sequences, originally termed v-bas and later renamed v-H-ras, homologous to the rat sequence in Ha-MSV (Andersen et al 1981). Unlike the other ras containing MSVs which express p21 v-ras, a 21,000 dalton protein, the rat v-H-ras gene acquired by Ra-MSV during its genesis was integrated such that it is expressed as a viral gag-ras fusion protein, p29 (Gonda et al 1982).

The basic definition of an oncogene has evolved from comparisons of the properties of genes which have been implicated in carcinogenesis. An oncogene has four properties 1- it is a eucaryotic gene, 2- it codes for a protein, 3- it has been conserved through evolution and presumably fulfills an essential physiological function in normal growth and development and 4- it has the potential to become a dominant oncogenic determinant (Bister and Jansen 1986). The normal cellular alleles of oncogenes have been designated as proto-oncogenes and the mutant alleles as active oncogenes (Bishop 1981, Weinberg 1982, Duesberg 1983). Comparisons between the cellular proto-oncogenes and the transduced viral oncogenes, have revealed several types of genetic mutations including truncation, point mutations, frame shift mutations and substitutions (reviewed in Bishop 1987).

Retroviral activation of a proto-oncogene can occur by several mechanisms including transcriptional effects and mutational effects. Insertion of a viral enhancer or promoter next to a proto-oncogene during proviral integration can cause increased proto-oncogene expression and tumorigenesis by inappropriate expression of a growth regulatory gene. The retrovirus can also cause mutations in proto-oncogenes during the process of integration or

replication (see Duesberg 1983 for review). Proto-oncogene activation by promoter insertion has been documented in the induction of avian B cell lymphomas by ALV. When this virus integrates adjacent to the c-myc proto-oncogene, transcription from the viral promoter through the c-myc gene causes enhanced expression of the c-myc gene product and neoplastic transformation (Hayward et al 1981). One of the many examples of activating mutations found in retrovirally transduced oncogenes is the Avian erythroblastosis virus acquired v-onc gene, v-erb B, which is a truncated form of the epidermal growth factor (EGF) receptor. The virally transduced receptor is missing a 30 amino acid external domain and is no longer responsive to EGF, thus causing transmission of an uncontrolled growth signal in the infected cell (Downward et al 1984).

Two conceptual models of oncogene activation, qualitative and quantitative, were described by Bishop in 1981 in an attempt to generalize the known mechanisms of all forms of oncogene activation, viral, chemical and spontaneous. The quantitative model invoked a "dosage hypothesis" in which disturbances of normal transcriptional or translational control can result in overproduction or inappropriate expression of an unaltered gene product which then results in uncontrolled cell growth. The above example of viral promoter insertion causing the over-expression of the normal c-myc protein provided support for this hypothesis in virus induced tumorigenesis. Chromosomal translocations between the c-myc proto-oncogene and the immunoglobulin loci in human Burkitt's lymphoma that result in deregulation of c-myc expression also provide support for this hypothesis (Leder et al 1983). Further evidence favoring this model comes from transfection studies in which overexpression of proto-oncogenes including mos (Blair et al 1981), ras (Chang et al 1982) and the EGF receptor (DiFiore et al 1987) under the control of retroviral promoters induces transformation. The qualitative model of oncogenesis states that activation of an oncogene is due to mutational change(s) in the coding domain of a proto-oncogene resulting in normal levels of expression of a functionally altered protein as in the previous example of v-erb-B.

The prevalence of specific point mutations activating ras proto-oncogenes in human tumors, as discussed below, provides an ample base of support for the qualitative model.

B. Transfection

DNA mediated transfection techniques were first established to induce the in vitro incorporation and expression of viral genes in eucaryotic cells, thus bypassing the necessity for viral infection. Using a DEAE dextran technique, Hill and Hillova (1971) first reported recovery of infectious virus by transfection with genomic DNA from cells infected with RSV. Graham and Van der Eb (1973) described an improved transfection technique based on calcium-phosphate-DNA co-precipitation. In 1978, a modification of the calcium phosphate transfection technique was shown to be sensitive enough to transfer single copy eucaryotic genes to recipient cells (Wigler et al 1978). Then, in 1979 Shih passaged the phenotype of a chemically transformed tumor to recipient NIH 3T3 cells by transfection with tumor DNA (Shih et al 1979). Soon several other laboratories reported similar results (Cooper et al 1980, Krontiris and Cooper 1981, Shih et al 1981, Pulciani et al 1982a). The transfection of tumor cell DNA, containing certain activated oncogenes, into NIH 3T3 recipient cells results in the morphological transformation of these normally contact inhibited cells into tumor cells. Activated oncogenes have been isolated from many tumors of non-viral origin using this method (Murray et al 1981, Perucho et al 1981, Pulciani et al 1982b, Padua et al 1984, Shimizu et al 1983, Cooper et al 1984, Eva and Aaronson 1985, Martin-Zanca et al 1986). Many of the tumor associated oncogenes isolated by transfection have turned out to be homologs of the retroviral oncogenes, particularly the members of the ras family (see Weinberg 1982, and Bishop 1987 for reviews). Some novel, oncogenes not related to those transduced by retroviruses have also been detected. The novel oncogene, trk, isolated from a human colon carcinoma, was shown to have been activated by a chromosomal

translocation that resulted in the fusion of a non-muscle tropomyosin gene with the transmembrane and tyrosine kinase domains of a receptor gene (Martin-Zanca et al 1986).

The first oncogene that was isolated by transfection of human tumor cell DNA was a homolog of the retroviral v-H-ras transforming gene of Ha-MSV (Der et al 1982, Santos et al 1982, Parada et al 1982, Goldfarb et al 1982). This T24 ras (c-Ha-ras-1) oncogene is activated by a point mutation in codon 12 of the coding sequence (Pulciani et al 1982b, Shih et al 1982, Tabin et al 1982, Taparowsky et al 1982, Capon et al 1983). Several strategies were employed by different investigators to isolate this oncogene. They all, however, used the NIH 3T3 cell transfection and focus formation assay (Shih et al 1979, Shih et al 1981) to detect the presence of a functional transforming gene in the donor DNA. The recipient NIH 3T3 mouse fibroblast cell line (Jainchill et al 1969) has an enhanced ability to take up and express exogenous DNA. When transformed, these normally flat, contact inhibited cells become rounded and refractile and show disorganized growth patterns resulting in the appearance of foci on top of the monolayer. The assay consists of immobilizing the tumor DNA in a calcium phosphate co-precipitate, pipetting the precipitate onto a sparse monolayer and observing the cells for the appearance of transformed foci over a period of 3 to 4 weeks. Since up to 10^4 kb of the donor cell DNA can be incorporated into a transfected cell (Wigler et al 1979, Perucho et al 1980), far more human DNA than that which is associated with the oncogene is usually found in the primary transformants. Secondary and even tertiary transfections may be required to randomly separate the transforming gene from irrelevant donor genes for subsequent cloning. Two types of genetic markers have been used to detect the presence of the donor oncogene in the recipient transfected cells: either repetitive sequences endogenous to the donor DNA, or a retrievable bacterial marker gene ligated to the donor DNA.

Several families of highly repeated DNA sequences are found interspersed throughout the human genome. One of the most abundant repeated sequence families is named "Alu" due to the presence of an Alu I restriction site found in most of its members.

This 300bp long repeat family is present at 300,000 to 900,000 copies in the human genome, with a random interspersed frequency averaging a few kilobases (Rinehart 1981, Kornberg and Rykowski 1988). Members of the Alu family were cloned into pBR322 and named the BLUR (Bam Linked Ubiquitous Repeat) plasmid series (Jelinek, et al 1980). Weinberg's group at the Massachusetts Institute of Technology was first to take advantage of this naturally occurring human sequence marker. They screened for the presence of human oncogenes in transfected mouse cell DNA by hybridizing Southern blots with the cloned BLUR 8 probe which does not cross hybridize with mouse repeated sequences (Shih et al 1981). As mentioned above, the primary transformants contained many co-integrated fragments of human DNA, whereas the secondary and tertiary transformants contained less human DNA, usually only that associated with the oncogene sequences. To clone the oncogene, they screened bacteriophage libraries of genomic DNA from NIH 3T3 cell secondary transformants for the presence of human repeated sequences using the same radioactively labelled BLUR 8 probe (Shih and Weinberg 1982). This method, and a slight variation using radioactively labelled total human genomic DNA, has since been used many times to isolate oncogenes from human tumor DNAs that induce focus formation in NIH 3T3 cells (Murray et al 1981, Pulciani et al 1982, Padua et al 1984, Eva and Aaronson 1985, Martin-Zanca et al 1986).

Michael Wigler's group at the Cold Spring Harbor Laboratory took a different, more generally applicable approach to finding a foreign gene in a genomic library of secondary transformants (Goldfarb et al 1982). They digested the human tumor DNA with a restriction enzyme which did not destroy its tumorigenicity, and ligated a bacterial marker gene, sup F, to each fragment before the primary transfection. This marker gene was then rescued from a crippled bacteriophage genomic library of secondary transformants as a tag for the presence of the donor DNA from the primary transfection. Theoretically, the bacteriophage vector cannot grow due to amber nonsense mutations in two essential genes. Since the

marker gene codes for an amber suppressor tRNA which will allow the phage to grow, only bacteriophage containing the marker gene should produce plaques when proper growth conditions and bacterial host are used. The few phage that grow under the restrictive conditions can then be screened for the presence of the oncogene by transfection assays on NIH 3T3 cells. In practice, however, the background reversion rate has been much higher than expected and tedious genetic analyses of many plaques was required to isolate the oncogene (M. Wigler, personal communication).

Once the transforming gene was cloned, it was used as a probe to isolate the homologous proto-oncogene from a human placental library and the restriction maps of the two genes were compared. No qualitative or quantitative differences could be found between the activated T24 ras oncogene and the c-H-ras proto-oncogene. The activating mutation was localized by construction of reciprocal hybrid genes between the normal proto-oncogene and the activated oncogene, and assaying for transforming activity by transfection of NIH 3T3 cells (Tabin et al 1982, Reddy et al 1982, Taparowsky et al 1982). The activating lesion was then determined by sequencing the smallest fragment that conferred transforming properties to the proto-oncogene. Sequence comparisons between the normal and mutant alleles revealed that a point mutation at codon 12 caused the substitution of a valine for a glycine in the T24 ras oncogene. This general series of experiments has become the standard method for determining the mechanism of activation of newly isolated ras oncogenes. Since two "hot spots" for activating point mutations have been identified in the ras genes, some newer methods of screening for these mutations only have been developed based on polymerase chain reaction amplification of exons one and two (Forrester et al 1987, Bos et al 1987). This form of genetic screening is fast and sensitive for the presence of mutations, however, the only mutations detected are those already known.

C. Newer Methods to Detect Transforming Genes

Since the majority of activated oncogenes detected by the focus formation assay on NIH 3T3 cells are members of the ras gene family that are activated by structural point mutations, several investigators have attempted to modify the assay and to look for alternative assays that are potentially more sensitive in the detection of other oncogenes. In an attempt to overcome the apparent bias of the focus formation assay to detect mutant ras genes, Blair et al (1982) described a bioassay based on transfection of NIH 3T3 cells followed by tumor formation in nude mice. However, the background spontaneous transformation rate of NIH 3T3 cells to the tumorigenic phenotype made the analysis of many tumors necessary. The increased sensitivity of the tumorigenicity assay for detecting oncogenes other than ras was shown by Keath et al (1984). They demonstrated that NIH 3T3 cells transfected with the myc oncogene became tumorigenic in nude mice without inducing significant morphological transformation. To overcome the high rate of spontaneous tumors generated by the tumorigenicity assay, Fasano et al (1984b) co-transfected limiting amounts of a dominant selectable marker plasmid with an excess of genomic tumor DNA. By pre-selecting the cells for the presence of the transfected marker gene before tumorigenesis testing, the background of spontaneous tumor formation was greatly reduced and the sensitivity of the assay was increased. This assay was sensitive enough to detect amplified normal ras genes according to Fasano (1984b), but in two other reports using similar techniques, the authors claimed that normal ras genes did not induce tumorigenesis (Bos et al 1985, Hirai et al 1987). Using a tumorigenesis assay, Wigler's group found a new oncogene they called mas, that had been activated by rearrangement in the non-coding sequences during transfection (Young et al 1986). Another gene created by rearrangements occurring during transfection, ret, was previously reported by Cooper's laboratory (Takahashi et al 1985).

Spontaneous and chemically induced tumorigenesis is known to be a multi-step process (reviewed in Land et al 1983b). Transformation of normal cells in culture into tumor cells also requires the cooperation of more than one activated oncogene (Land et al 1983a, Newbold et al 1983, Ruley et al 1983). Oncogenes were placed in two complementation classes, the ras- or the myc- class, based upon cooperation in transforming primary cells, usually rat embryo fibroblasts. NIH 3T3 cells are pre-neoplastic and only require the action of a single activated oncogene to become tumorigenic (reviewed in Cooper 1982). In several instances more than one activated oncogene have fortuitously been detected in the same tumor using NIH 3T3 cell transfection assays (Murray et al 1983, Fasano et al 1984b).

D. The Mammalian ras Oncogenes

Following the discovery that the human oncogene isolated from T24 cells was homologous with a viral ras oncogene (Parada et al 1982), research into the characterization of Ras proteins and ras induced transformation has exploded. The impetus to study ras has been boosted further with the realization that up to 40% of human tumors contain activated ras genes (reviewed in Barbacid 1987). Using the polymerase chain reaction to detect activated ras genes instead of one of the bioassays, two groups showed that a high proportion (40%) of colon carcinomas have ras point mutations, usually K-ras (Forrester et al 1987, Bos et al 1987). Even though the ras literature is vast, the function(s) of the mammalian ras genes remain largely speculative. Of the three mammalian ras genes, H-ras, K-ras and N-ras, the latter is the only one without a viral homolog. N-ras was first discovered by transfection of DNAs from human neuroblastoma, leukemia and sarcoma cell lines (Shimizu 1983, Hall 1983). All three genes have similar intron-exon boundaries and the p21 proteins are highly homologous in several regions suggesting divergence from a common ancestral gene. A series of monoclonal antibodies generated against viral Ras proteins show varying cross-reactivities between the three proteins (Furth et al 1982) and

several of these antibodies have been used extensively for p21 detection and biochemical characterization.

1. Biochemical Characterization

The p21 Ras proteins bind guanine nucleotides (Shih et al 1980) and are structurally similar to the signal transducing G proteins. The possibility that they participate in signal transduction was reinforced by their localization at the inner surface of the cytoplasmic membrane (Willingham et al 1980). The Ras proteins have a low GTPase activity that is decreased in oncogenic mutants (Gibbs et al 1984). All three ras oncogenes have been activated by point mutations at codons 12, 13, and 61 by either viruses, chemical carcinogens or in human tumors (reviewed in Barbacid 1987). Several other potential activating mutations have been identified by in vitro mutagenesis studies (Fasano et al 1984a, Sigal et al 1986a). All mutations that activate the oncogenic potential of Ras proteins effect the binding or GTPase activity of Ras such that the GTP bound form is favored, therefore it is assumed that the GTP bound form is the active form of the Ras protein. The retroviral v-H-ras also has a mutation that substitutes a threonine for an alanine at position 59 which becomes auto-phosphorylated (Shih et al 1980); the normal p21 Ras is not phosphorylated.

Two tools have greatly facilitated the characterization of Ras proteins: production of large quantities of functionally active Ras p21 in E.coli (Lautenberger et al 1983, Poe et al 1985), and of monoclonal antibodies that bind to specific epitopes of the p21s. Micro-injection of physiological amounts of purified v-H-ras p21 into NIH 3T3 cells caused morphological transformation and thymidine incorporation; micro-injection of a larger quantity of the normal protein was required for transformation (Stacey and Kung 1984). Of the monoclonal antibodies that were made to viral Ras proteins, Y13-259 has been used most extensively to detect and characterize Ras p21's since it cross-reacts with all three mammalian Ras proteins (Furth et al 1982, Srivastava et al 1985). Monoclonal antibody

Y13-259 binds to 6 amino acids within a conserved epitope, aa 63-73 (Sigal et al 1986b). Antibody binding to p21 interferes with GTP binding, probably by steric hinderance. Microinjection of this monoclonal anti-p21 inhibits serum stimulation of cell growth and blocks NIH 3T3 cells in G0 supporting the idea that the ras proto-oncogene is involved in growth control (Mulcahey et al 1985). Micro-injection of ras transformed cells with a monoclonal antibody specific for the codon 12 mutation caused reversion of the transformed phenotype (Feramisco 1985), thus proving that ras activation is required to sustain transformation. In an attempt to address the potential signal transduction mechanism of Ras proteins, monoclonal Y13-259 was injected into cells transformed with oncogenes known to be active in different parts of the cell. Antibody injection blocked the entry into S phase of cells transformed by growth factor receptor oncogenes such as fms or oncogenes that associate with the cell membrane such as src and fes, but had no effect on the cytoplasmic oncogenes mos and raf (Smith et al 1986). These results support the hypothesis that p21 Ras is involved in signal transduction of at least some stimuli for growth. Micro-injection experiments with ras proteins and antibodies were recently reviewed by Bar-Segi (1989).

Using EM immunocytochemistry, Willingham et al (1980) localized Ras proteins to the inner surface of the cytoplasmic membrane. Early on it was realized that Ras was synthesized as a precursor and was processed in some way that allowed membrane association (Shih et al 1982). The cysteine at position 186 was shown to be required for ras mediated transformation and for localization to the plasma membrane (Willumsen et al 1984). It was first thought that palmitoylation of the amino terminal cysteine was the modification required for membrane association (Chen et al 1985), but only a small portion of Ras is acylated. More recent experiments have shown that the primary translation product, pro-Ras, goes through several post-translational modifications, including addition of a farnesyl isoprenoid moiety to Cys 186 (Casey et al 1989), carboxy-terminal trimming of the last three amino acids and methylation of the new carboxy terminus, Cys186 (Gutierrez

et al 1989). These irreversible changes are followed by palmitoylation of cysteines within the variable region adjacent to Cys186. Since only a small portion of the Ras proteins are palmitoylated it has been speculated that this final modification may have a role in directing the protein to a specific region of the membrane for signal transduction (Hancock et al 1989).

The Ras proteins have two GTP binding domains, aa 106-120 and 152-165 (reviewed in Barbacid 1987), and an effector domain, aa 35-40 (Sigal et al 1986b). At least one protein is now known to bind to the effector domain- the GTPase Activator Protein, GAP (Adari et al 1988). The GAP protein is responsible for activating the GTPase activity associated with normal p21, but it has no effect on the codon 12, 59 or 61 ras mutants associated with transformation (Trahey and McCormick 1987). Therefore GAP is thought to have two activities in relation to Ras, 1) it specifically binds to Ras p21 and may act as the downstream signal transducer when Ras is in the GTP bound state, and 2) GAP activates the GTPase activity of Ras inducing dephosphorylation of the bound GTP, thus turning off its own activation signal. All ras mutants that do not bind GAP are non-functional and the 12, 13, and 61 mutants that bind GAP are not susceptible to the GTPase activating activity of GAP and therefore are always in the active configuration (reviewed in McCormick 1989).

2. Gene Structure and Expression

The three members of the ras family share a basic genomic structure of 4 coding exons with identical intron-exon boundaries suggesting that they may have emerged from a common ancestral gene (Taparowski et al 1983). A large portion of the 5' non-coding region of all three genes is located on a separate -1 exon, and many ras genes have upstream ATG initiation codons. The significance of this deviation from the usual case of initiation at the ATG closest to the mRNA CAP is not yet understood (Kozak 1989). The promoter regions of the ras genes are also unusual. Instead of the usual TATA and CAAT sequences, the ras promoters contain regions high in G + C nucleotides (reviewed in Lacal et al 1988). These

GC boxes were originally found associated with housekeeping genes, but since these same motifs are also associated with the promoter of the EGF receptor it is speculated that this type of promoter may be associated with certain genes involved in growth regulation (Ishii et al 1985).

The promoter of the human c-K-ras 2 gene has 4 repeats of G + C rich motifs and no TATA or CAAT box. It is the largest member of the ras family due to very large introns and alternative 3'ends (McGrath et al 1984, McCoy et al 1984). An alternative fourth exon, termed 4b, codes for a functional p21 that has no cysteines in the variable region resulting in the only form of p21 that is not palmitoylated (Casey et al 1989). The functional significance of the two forms of K-ras p21 is not yet clear. This gene is frequently activated by point mutations in human colon and lung carcinomas (McCoy et al 1983, reviewed in Kahn et al 1987). Due to its large size, approximately 45 kb, it has been very difficult to isolate activated K-ras oncogenes from tumor DNAs. Several investigators have used the polymerase chain reaction (PCR) to detect mutant K-ras genes in tumor DNAs. This technique is more sensitive than transfection and not dependent on the intactness of the DNA sample, but is limited by its ability to detect only known mutations. Using PCR, the frequency of K-ras mutations was found to be very high in human colorectal tumors (Forrester et al 1987, Bos et al 1987). Using a combination of PCR amplification and RNase A mismatch cleavage, Almoguera et al (1988) showed that codon 12 of c-K-ras was mutant in 21 of 22 pancreatic carcinomas tested.

The N-ras gene has been most often associated with tumors of myeloid origin even though it was first cloned from a neuroblastoma cell line (Taparowsky et al 1983). Using a nude mouse tumorigenesis assay, point mutations in codon 13 were detected in acute myeloid leukemias (Bos et al 1985, Hirai et al 1987). Although codon 13 mutations had been shown to activate the oncogenic potential of p21 Ras by site directed mutagenesis, this was the first isolation of a codon 13 mutation in a natural tumor type. The translated portions of the gene have been completely mapped and sequenced, and the exon structure in the 3'

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end of the gene was found to be unique (Hall and Brown 1985). In addition to the 4 coding exons and the -1 non-coding exon, N-ras has a very small 3' non-coding exon, exon 5, followed by a second non-coding exon, exon 6, which contains two alternative polyadenylation sites. The first polyadenylation site is not efficiently recognized, resulting in the transcription of two mRNAs of 2 and 4.3 kb, the longer being a read through product of the shorter. Like the other ras genes, the promoter region of N-ras consists of regions high in G + C nucleotides with 4 repeats of the GGGCGG consensus for binding of the transcription factor Sp1 (Dyran and Tjian 1983), called GC boxes. Human N-ras mRNA has two upstream ATGs; the second ATG, which is conserved with guinea pig N-ras (Doniger 1987), is in a good context for initiation of translation of a short open reading frame that terminates 18 nucleotides 5' of the p21 initiation site (Hall and Brown 1985).

c-H-ras is the smallest of the three ras genes, the active human gene being approximately 4.5 kb (Barbacid 1987). Like K-ras and N-ras, this gene has 4 coding exons, and a -1 exon representing most of the 5' untranslated region (UTR). It has recently been noted that the 3'UTR of the human gene may be alternatively spliced since some cDNA clones contain a deletion 3' of the translation termination site (Cohen et al 1989). H-ras activation by specific point mutations has consistently been found in several animal models of carcinogen induced tumorigenesis (reviewed in Barbacid 1987), including induction of mammary tumors in rats (Zarbl et al 1985) and in multistage mouse skin carcinogenesis models (Bizub et al 1986). H-ras activation may be an early event in the skin carcinogenesis model since mutations have been detected in pre-cancerous lesions (Balmain et al 1984). H-ras was also the first human oncogene identified by transfection of human tumor DNA into NIH 3T3 cells, and therefore has been the most studied of the three members of this gene family. Even so, studies on the control of ras gene expression have lagged far behind those on its biochemical characterization.

The promoter regions of the human (Ishii et al 1985, Honkawa et al 1986), rat (Damante et al 1987), and mouse (Brown et al 1988) c-H-ras gene have been identified and

sequenced. The H-ras promoter contains 5 GC boxes and was shown to bind the Sp1 transcription factor at these sites by DNase I footprint analysis of the human gene (Ishii et al 1986). The published mRNA start sites of the human gene are conflicting (Ishii et al 1985, Honkawa et al 1986) but the rat and mouse have 3 similar start sites (Brown et al 1988). Portions of the mouse 5'UTR and putative promoter induced CAT production when placed 5' of a CAT gene. By comparing the relative CAT activity of 3 deletion constructs the authors concluded that an enhancer is located in the first intron between exon -1 and 1 of the mouse H-ras gene (Brown et al 1988). Using CAT assays, promoter and enhancer like activity has also been demonstrated in a 0.8 kb SstI genomic fragment that includes the -1 exon of the human H-ras gene (Spandidos and Riggio 1986).

Recently, a group at Genentech has identified a point mutation in the 5' splice junction of an alternative exon, IDX, within intron D (between exons 3 and 4) of the human T24 ras, c-H-ras-1 oncogene. This mutation causes increased transforming potential of the oncogene (Cohen and Levinson 1988, Cohen et al 1989). Translation of the alternatively spliced mRNA putatively results in a truncated form of H-ras. Whether this truncated protein has a function or is actually made has not yet been proven. Placement of the IDX sequence within an intron caused reduced expression of ras and of an unrelated reporter gene (Cohen et al 1989). Since the normal 5' splice site of IDX is not consensus, a mechanism of defective processing of the alternatively spliced mRNA was proposed for the observed negative effect on expression. Previous studies of requirements for 5' splice junctions have shown that deviation from consensus can interfere with the second step of splicing and result in defective intermediates that are lost during processing (reviewed in Aebersold and Weissmann 1987). Cohen et al (1989) demonstrated that the splice junction mutation in T24 ras abolished alternative splicing of the IDX sequence by rendering its poor 5' splice site unrecognizable. Point mutation of the T24-ras IDX splice junction back to normal reduced the focus forming activity of T24 ras 10 fold without significant accumulation of the alternatively spliced mRNA, supporting the notion that the alternatively spliced transcripts

are defectively processed. Further point mutations to create a 5' splice site that matched consensus abolished the transforming activity of T24 ras, and resulted in accumulation of the alternatively spliced mRNA, possibly by correction of the defective splicing. As demonstrated by these experiments, defective processing of transcripts containing the IDX sequence may decrease gene expression by reducing the percentage of transcribed RNAs that can be translated. Since the H-ras protein is relatively stable with a half-life of 20 hours (Ulsh and Shih 1984), defective processing may be a mechanism used by the cell to prevent overexpression of this potentially oncogenic protein. The human T24 ras(c-H-ras 1) oncogene has both a dominant mutation at codon 12, and a mutation in the 5' splice junction of the IDX that increases the transforming potential of the gene in transfection experiments (Cohen and Levinson 1988). Earlier studies into the mechanism of activation of T24 ras, however, demonstrated that the mutant p21 protein was not significantly (less than 3 fold) overexpressed in the T24 tumor cells from which it was derived (Tabin et al 1982, Der et al 1982). Other mechanisms must also be involved in modulating the expression of p21 in the cell.

The ras proto-oncogene is expressed at low levels in all cells (Furth et al 1987), and at all stages of development (Muller et al 1982). Elevated levels of p21 have been detected in some non-proliferating tissues such as brain, heart and some terminally differentiated endocrine cells (Furth et al 1987). In addition to its function as a signal transducer in response to growth stimuli, p21 may also function in signal transduction of differentiation stimuli in certain cell types. In agreement with this hypothesis micro-injection of the codon 12 substituted ras p21 (Bar-Sagi and Feramisco 1985), or the normal p21 pre-incubated with the GTPase resistant GTP γ S (Satoh et al 1987) induced terminal differentiation of PC12 pheochromocytoma cells. Infection of human medullary thyroid carcinoma cells with Ha-MSV also induced differentiation into more normal C type thyroid cells (Nakagawa et al 1987). Therefore the ras proteins may have a dual function as a signal transducer for

normal cellular proliferation and in certain cells Ras may be involved in the activation of a pathway that induces terminal differentiation.

CHAPTER II

MATERIALS AND METHODS

A. Purification of DNA

1. Isolation of DNA from tumors

Insulinoma tumor tissue was obtained either as fresh tissue on ice from the surgical unit at the NIH clinical center or as tissue frozen on dry ice and stored at -70°C from M. Linehan (NCI). Tumor tissue was obtained from nude mice, frozen on dry ice and stored at -70°C until use. Tumor tissue was weighed, washed and minced into small pieces. Genomic DNA was extracted by a modification of the method of Wigler et al (1979). The minced tissue was homogenized in a dounce homogenizer in TNE buffer (refer to Appendix A for solutions), and SDS was added to a final concentration of 0.5% to lyse the cells. Lysates were treated with proteinase K (200 $\mu\text{g}/\text{ml}$, Boehringer Mannheim, Indianapolis, IN) at 56°C overnight. The crude DNA was gently extracted with phenol: chloroform (1 part redistilled nucleic acid grade phenol, BRL, Gaithersburg, MD, to 1 part reagent grade chloroform, J.T. Baker Chemical Co., Phillipsburg, NJ, containing 1/24th volume isoamyl alcohol, hereafter referred to as Phe: CHCl_3). After a final extraction with chloroform: isoamyl alcohol (24 parts reagent grade chloroform, J.T. Baker, to 1 part reagent grade isoamyl alcohol, J.T. Baker, hereafter referred to as CHCl_3 :IAA), the DNAs were precipitated with 0.2 M NaCl and two volumes of 95% ethanol and spooled out of solution on sterile glass rods. The spooled DNAs were washed with 70% ethanol, air dried, and dissolved in TE buffer slowly with refrigeration. DNAs were then incubated with RNase (60 U RNase A, 60 $\mu\text{g}/\text{ml}$ RNase T1, see appendix A for preparation of stock solution) at 37°C for 30 minutes, followed by treatment with proteinase K at 200 $\mu\text{g}/\text{ml}$ for 30 minutes at 56°C . The DNAs were again extracted and spooled out of ethanol. The purified DNAs were dissolved in TE buffer and the approximate concentrations were determined by absorbance at 260 nm using an

extinction coefficient of $20 \text{ OD}_{260} = 1 \text{ mg dsDNA}$. The absorbance ratio of 260/280 was also determined and was routinely between 1.7 to 1.9.

2. Isolation of DNA from Tissue Culture Cells

DNA was extracted from pellets of tissue culture cells in essentially the same manner. Cells were trypsinized from two- 150 cm^2 tissue culture flasks by washing each of the monolayers with 5 ml of 0.25 % trypsin, 5 mM EDTA in saline (NIH Media Unit), followed by incubation with 1 ml per flask of fresh trypsin solution until the cells released from the monolayer. The cells were diluted with growth medium (see transfection of NIH 3T3 cells) and the single cell suspension was centrifuged at 1000 g for 5 minutes. The cell pellet was washed twice with cold DPBS (Dulbeccos PBS, without calcium and magnesium, Whittaker Bioproducts, Walkersville, MD) by gentle resuspension and centrifugation. The final cell pellets were resuspended in 10 ml TNE buffer and processed as for tumor DNA. The final yield of DNA was approximately 2 mg/g tissue and $300 \mu\text{g}/10^7$ tissue culture cells.

3. Purification of Plasmid DNA

The procaryotic-eucaryotic marker plasmid, pSV2-neo, was obtained from a glycerol stock in *E.coli* HB101. An isolated ampicillin resistant colony was inoculated into 3 ml Luria-Bertani broth (LB, Digene, Silver Spring, MD) containing 100 $\mu\text{g}/\text{ml}$ ampicillin (LB + AMP) and the culture was grown overnight at 37°C with shaking. One ml of the overnight culture was added to 25 ml LB + AMP and shaken at 37°C until the OD_{600} was approximately 0.6. This log phase culture was added to 500ml pre-warmed LB + AMP and shaken at 37°C for 2.5 hours. Chloramphenicol was added (2.5 ml of a 34 mg/ml solution in ethanol) and the culture was shaken overnight to amplify the plasmid (Clewell, 1972). The cells were harvested by centrifugation in a Sorvall RC5 with an HS4 rotor at 4,000 g for 20 minutes. The plasmid DNA was isolated by a large scale modification of the Holmes and Quigley (1981) boiling procedure. The pellet was resuspended in 25 ml STET (see appendix A),

transferred to an Oakridge tube (Beckman, Paolo Alto, CA) and frozen on dry ice. The cells were lysed by rapid thawing and addition of 1 ml freshly prepared lysozyme (10 mg/ml in 20 mM Tris, pH 8.0). The suspension was boiled in a water bath for 1 minute and cooled on ice. The denatured protein and chromosomal DNA were pelleted in a Beckman LS75 ultracentrifuge with a Ti60 rotor at 25,000 rpm for 45 minutes at 4°C. The supernatant was carefully removed to a 50 ml polypropylene centrifuge tube (Falcon, Becton Dickinson, Lincoln Park N.J.), proteinase K was added to a final concentration of 200 µg/ml, and incubated at 56°C for 30 minutes. The plasmid preparation was extracted once with Phe:CHCl₃ and once with CHCl₃:IAA being careful not to disturb the white interface. The phases were separated in both extractions by centrifugation at 4000 g for 20 minutes in a Sorvall RC-3 at 4°C. One volume of propanol was added to the aqueous phase from the last extraction and incubated at -20°C overnight to precipitate the plasmid. The precipitate was pelleted in a Sorvall RC-3 centrifuge at 4°C for 30 minutes. The pellet was washed with 5 ml of 80% ethanol and dried under vacuum. The plasmid DNA was resuspended in 5 ml TE buffer and incubated with 50µl RNase stock solution (see appendix A) at 37°C for 30 minutes. Proteinase K was added to a final concentration of 200 µg/ml and the incubation was continued for 30 minutes longer. The aqueous phase was again extracted with Phe:CHCl₃, and CHCl₃:IAA, as mentioned above. Plasmid DNA was precipitated with 0.5 volume of 7.5 M ammonium acetate and 2 volumes of 95% ethanol at -20°C for at least 2 hours. The precipitate was pelleted, washed and dried as above, and resuspended in approximately 500µl TE buffer.

The plasmid DNA was separated from degraded low molecular weight RNA by column chromatography. A 30 cm column of Sephacryl S-1000 (Pharmacia, Piscataway, NJ) was equilibrated with 20 mM Tris, pH 7.2, 1 M NaCl, 1 mM EDTA. The plasmid was layered over the column bed and the eluate was monitored for absorbance at OD₂₆₀. Fractions were collected and checked for the presence of the plasmid by electrophoresis in a 1 % agarose gel (see appendix 1 for buffer). All positive fractions were pooled and precipitated with 2

volumes of 95% ethanol at 4°C overnight. The pellets were washed and dried as above and resuspended in 200 µl TE buffer. The DNA concentration was determined by OD₂₆₀, as with genomic DNA.

A new method of plasmid purification was used for sequencing the subclones of the phage inserts, preparing chimeric plasmids, and in vitro transcription. The method is based on a modification of the alkaline lysis method of Birnboim and Dolby (1979), followed by adsorption of plasmid DNA to a QiagenTM column (Qiagen, Studio City, CA). The degraded RNA and protein contaminants were washed through the column and the pure plasmid DNA was eluted in 1.2 M NaCl, 50 mM 3-[N-morpholino]propanesulfonic acid (MOPS), 15 % ethanol. The Qiagen columns were purchased as complete kits, including buffers, and were used according to the instructions of the manufacturer. The eluted plasmid DNA was precipitated with 0.7 volumes propanol at room temperature. After washing the precipitate with 70 % ethanol, the DNA was resuspended in TE buffer and re-precipitated with 0.5 volumes of 7.5 M ammonium acetate and 2 volumes of ethanol at -20°C for 2 hours. The precipitate was pelleted, washed and dried as above. The pure plasmid DNA was resuspended in TE buffer and the concentration was determined by OD₂₆₀ as above. The purity of these plasmid DNA preparations was equivalent to cesium chloride ethidium bromide gradient purified plasmids with a preparation time of hours instead of days.

4. Purification of Lambda Phage DNA.

Phage clones were grown in the respective E.coli host as mentioned under the library construction sections. Minipreparations of phage DNA were prepared from plate lysates by adsorption with lambdasorbTM (Promega Corp., Madison, WI). Two- 100 mm petri dishes (1025, Falcon) or one- 150 mm plate (3025, Falcon) were overlayed with 6 ml (total) warm LB top agarose containing 300 µl freshly prepared host bacteria infected with 10⁵ phage. The agar was allowed to solidify at room temperature, then the plates were incubated at 37°C until complete lysis occurred, usually 8 hours to overnight. The phage were harvested by

layering 5 ml phage dilution buffer (TM, see appendix B) over the plate(s) and allowing the phage to diffuse into the buffer during a 1 hour incubation in the cold with gentle rocking. The buffer was collected and replaced with fresh buffer for one additional hour. The phage were pooled and the total volume was adjusted to 10 ml with TM buffer. The phage suspensions were shaken with a few drops of chloroform and the debris was pelleted by centrifugation at 4,000 g in a Sorvall RC3. The supernatant bacteriophage were adsorbed to 100 μ l lambdasorbTM for 30 minutes on ice with intermittent mixing in a polypropylene tube (2059, Falcon). The lambdasorb-phage precipitate was collected by centrifugation at 10,000 g for 30 minutes at 5°C. The pellet was carefully resuspended in 1 ml TM buffer and transferred to a 1.5 ml Eppendorf tube. The lambdasorb was re-pelleted in a microfuge for 1 minute, and washed again in 1 ml TM buffer. The final pellet was resuspended in 0.5 ml TE buffer, EDTA was added to 10 mM and the phage DNA was released by incubation at 70°C for 5 minutes. The debris was pelleted in a microfuge for 10 minutes and the supernatant was extracted once with Phe:CHCl₃ and once with CHCl₃:IAA. The DNA was precipitated with 0.5 volumes 7.5 M ammonium acetate and 2 volumes ethanol at -20°C for 2 hours. The DNA precipitate was pelleted in a microfuge, washed with 70 % ethanol, dried under vacuum, and gently resuspended in 200 μ l TE buffer. Residual RNA contamination was removed by digestion with RNase (see appendix A for stock solution) at 37°C for 15 minutes. The DNA was then re-extracted and precipitated as above. Phage DNAs prepared by this method were used for restriction mapping and subcloning.

Liquid lysates were grown of selected phage clones to make large amounts of purified phage DNA for transfection studies. A 10 ml culture of host bacteria, eg. E.coli LE392 was grown overnight and the OD₆₀₀ was determined. An OD of 1.8 = 1.4×10^9 cells. The equivalent of 10^{10} cells were pelleted and resuspended in 3 ml SM buffer. The bacteria were inoculated with 10^{10} phage and incubated without shaking at 37°C for 20 minutes for phage attachment. The infected cells were inoculated into 500 ml NZCYM broth (see appendix B) and shaken in a rotory shaker at 200 revolutions per minute for approximately 5

hours at 37°C until the culture lysed. Chloroform (5 ml) was added to help complete the lysis and the culture was shaken for 15 minutes. Sodium chloride was added to 0.5 M and the culture was shaken for 15 more minutes. The bacteriophage were purified by a modification of the method first described by Yamamoto et al (1970). The cellular debris was pelleted by centrifugation at 7,000 g for 15 minutes at 5°C and the phage in the supernatant were precipitated with 10% polyethylene glycol (PEG 8000, Baker) on ice for 1 hour. The precipitate was collected by centrifugation at 7,000 g for 15 minutes at 5°C. The precipitated phage were gently resuspended in SM buffer using a wide bore pipet and the polyethylene glycol was extracted from the bacteriophage suspension by shaking with with an equal volume of chloroform. The phases were separated by centrifugation at 3,000 g for 15 minutes at 5°C and the aqueous phase containing the phage particles was used for further purification.

The phage were banded on a cesium chloride step gradient. The resuspended phage were diluted to 20 ml with SM buffer and half was layered over each of two step gradients consisting of 13 ml 1.4 g/ml cesium chloride in TM over 13 ml 1.6 g/ml cesium chloride in TM in polyallomer ultracentrifuge tubes (326823, Beckman). The gradients were run in an SW28 rotor at 20,000 rpm, for 18 hours at 20°C. The phage bands were collected, pooled, and desalted by column chromatography on pre-packed Sephadex columns (PD10, Pharmacia) equilibrated with TM buffer. The desalted phage were digested with crude DNase (1 µg/ml, Sigma), and RNase (6 µg/ml T1, Sigma) at 37°C for 30 minutes. Proteinase K was added to 200 µg/ml, EDTA to 10 mM, SDS to 0.5%, and the lysed phage were incubated at 56°C for 30 minutes. Phage DNA was extracted with Phe:CHCl₃ and CHCl₃:IAA as mentioned for other DNA preparations and was precipitated by the addition of NaCl to 0.2 M and 2 volumes of ethanol. The precipitated DNA was spooled out of solution on a small glass rod, washed with 80 % ethanol and air dried. The purified DNA was resuspended in TE buffer and the concentration was determined as above.

B. Transfection of NIH 3T3 Cells

1. Maintenance of NIH 3T3 Cells

NIH 3T3 cells (Jainchill et al 1969) were obtained from Mariano Barbacid (NCI). Proper maintenance of these cells is critical for reproducible tumorigenesis and focus formation results. The cells were immediately grown in several 175cc- T flasks and frozen in liquid nitrogen at one passage after receipt in aliquots of approximately 10^6 cells. The cells were trypsinized as described previously at 80 % confluence and washed with growth medium. The cells were centrifuged at 1000 g for 5 minutes, resuspended at approximately 10^6 cells/ml in freezing medium, and 1 ml aliquots were frozen first at -70°C in 1.5 ml vials (Nunc, Denmark) and after 2 days Equilibration, transferred to liquid nitrogen. Freezing medium consisted of 10 % dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO), 15 % fetal bovine serum (FBS, Gibco, Grand Island, NY), and 75% Dulbecco's modified Eagles medium (high glucose DMEM, Whittaker Bioproducts). For each transfection experiment an aliquot of cells was quickly thawed in a 37°C waterbath with constant agitation, and washed by dilution with 10 ml growth medium and centrifugation at 1000 g. The cells were then resuspended in growth medium and plated into 2- 75cc flasks at 2/3, and 1/3 of the frozen cells per flask. Growth medium was DMEM with 100 mg/ml penicillin G, 135 mg/ml streptomycin sulfate, 2 mM glutamine, and 10% heat inactivated calf serum (Colorado Serum Co., Denver, CO). The cells were observed daily and the flask that was 80 to 90 % confluent on the day before transfection was used as seed stock.

2. Calcium Phosphate Mediated DNA Transfection

NIH 3T3 cells were co-transfected with tumor or cellular DNA and pSV2neo using a modification (Wigler et al 1979) of the procedure of Graham and Van der Eb (1973). The procedure was obtained from Mariano Barbacid's laboratory (NCI). Briefly, on the day before the transfection, NIH 3T3 cells were plated at 10^5 cells per 100 cm^2 dish (Falcon) in

growth medium. On the morning of the transfection day, the plates were observed for the presence of a sparse even monolayer, and the medium was changed. The DNAs to be transfected (usually 600 ng pSV2neo and 80 to 100 µg tumor DNA for 2 plates) were first co-precipitated with 0.2 M NaCl and 2 volumes of 95% ethanol for 30 minutes on dry ice. The DNAs were pelleted and washed with 80% ethanol and dried under vacuum. The DNAs were treated as sterile at this point, and resuspended in sterile distilled water (0.25 ml/plate). A calcium phosphate-DNA co-precipitate was prepared by the addition of 0.25 ml/plate of 0.5 M CaCl_2 to the resuspended DNAs, followed immediately by dropwise addition of the DNA mixture to an equal volume of 2X HNP buffer (49 ml of 50 mM Hepes, pH 7.1, 280 mM NaCl mixed with 1 ml of 70mM sodium phosphate, pH 7.1 just before using) with constant mixing. The co-precipitate was allowed to form for 30 to 60 minutes at room temperature. This fine precipitate was then gently resuspended and pipetted onto the NIH 3T3 cell monolayers. Twelve hours later the media were changed, and 2 hours later changed again to make sure that all of the precipitate was washed off.

3. Focus assay

If the transfection was for a focus assay, then pSV2neo was omitted and the transfected cells were fed with maintenance medium (DMEM with 5% CS). The cells were re-fed at 3 to 4 day intervals and observed for the formation of foci of cells growing on top of the growth arrested monolayer. Negative plates were discarded at 6 weeks. Foci were picked using trypsin soaked filter paper discs. Briefly, the location of the foci were marked on the bottom of the plate and the monolayer was washed with 4 ml DPBS, which was then removed. Small discs of Whatman 3MM paper that had been cut with a hole punch and autoclaved, were soaked in trypsin EDTA solution and using flamed forceps the soaked paper was placed on the focus for approximately 30 seconds. The paper disc was then lightly rubbed back and forth to dislodge the cells. The paper and the trypsinized cells were then transferred to a 24 well plate containing 2 ml maintenance medium per well. The cells

were allowed to attach overnight and the paper was removed and medium changed the next day.

4. Tumorigenesis Assay.

If the transfection was for tumorigenesis assays, then the co-transfected cells were incubated for 18 to 20 hours after washing off the precipitate to allow for the expression of the transfected DNA. For sib-selection of primary transfections, each dish of cells was split 1 to 10 and fed with selection medium containing 300 $\mu\text{g/ml}$ G418 (Gibco) to select for the presence of the co-transfected plasmid, pSV2neo. Since the co-transfected genomic donor DNA was in excess (300 ng plasmid to 30-50 μg genomic DNA), all cells expressing the selectable marker should also have integrated donor DNA sequences. The medium was changed every 3 to 4 days until distinct colonies of resistant cells formed (approx. 2 weeks). The efficiency of transfection of pSV2neo was approximately 3 to 5 thousand colonies per 300 ng plasmid per 10^6 cells. Ten pools of 300-500 colonies each (usually one plate from the 1 to 10 split) were transferred to a 175 cc flask plus a 75 cc flask each and fed with selection medium. For secondary transfections, each plate of co-transfected cells was split 1 to 8 and all of the colonies were pooled before transfer to larger flasks as above. For co-transfection of purified phage clone DNAs, the pSV2neo concentration was reduced to 200 ng/plate using 500 ng/plate of each clone and NIH 3T3 cell carrier DNA to total 20 μg DNA/plate. Each of these plates were split directly into T 175 flasks for selection since most G418 resistant cells also expressed the transfected oncogene.

The resistant cell pools were allowed to grow to 80 % confluence for injection into nude mice. At the time of injection, an aliquot of the pooled cells was also frozen in liquid nitrogen. The cells were prepared for injection by trypsinization and washing with 10 ml growth medium. The cells were washed again with DPBS and counted with a hemocytometer. The cells were then pelleted and resuspended in DPBS to a final concentration of 2×10^7 cells/ml. For primary transfections, 10 independent pools were

prepared for injection (5 mice/ pool). One month old female NIH Swiss nu/nu mice were injected subcutaneously over the right rear leg with 0.1 ml of cells. The mice were then observed for tumor production at the site of inoculation for 16 weeks. Mice injected with NIH 3T3 cells co-transfected with positive control DNA from T24 human bladder carcinoma cells (c-H-ras, ATCC# HTB 4) routinely developed tumors within 1 to 2 weeks. Mice injected with NIH 3T3 cells transfected with pSV2neo alone produced tumors at a very low frequency and then only after prolonged incubation (14-16 wks). Tumor growth was recorded and when the tumors reached 15-20 mm diameter the mice were sacrificed and the tumor tissue was frozen at -70°C for further analysis as per animal use protocol number R-89-2. Mice were also inspected for the presence of metastases. No metastatic tumors were seen.

C. Detection of transfected DNA

1. Screening for Human Repeated Sequences

Southern blots of digested genomic DNA from primary and secondary transformants were hybridized with probes for repeated sequences unique to the donor transforming DNA. Genomic DNAs (10 µg/lane) from primary and secondary NIH 3T3 cell tumors were digested to completion with 5 Units/µg of the restriction endonuclease, EcoRI (BRL, high concentration) at 37°C for 2 to 4 hours and electrophoresed in 0.8 % agarose (BRL) gels (see appendix 1 for Universal restriction enzyme buffer and electrophoresis buffer). The position of the molecular weight markers (1 kb ladder, BRL) were marked by stabbing the gel with a needle dipped in India ink, and the digested DNAs were transferred to a charged nylon membrane (Nytran, Schleicher and Scheull, Keene, NH) by a modification of the method of Southern (44). Briefly, the gels were soaked in 0.25 M HCl, to nick the high molecular weight DNA, for 15 minutes with rocking. The gels were rinsed in distilled water and soaked in denaturing solution (see appendix A) 2 times for 15 minutes each with rocking. The gels were rinsed in distilled water and transferred to neutralization solution for

two additional 15 minute incubations and finally soaked in transfer buffer, 10 X SET.

Capillary transfer (Maniatis et al 1982) of the DNA from the gel to the membrane was allowed to proceed overnight.

The DNAs were initially fixed to the blots by baking at 80°C under vacuum for 2 hours. The method was changed after determining that UV cross-linking with the StratalinkerTM (Stratagene, La Jolla, CA), set to the auto-crosslink mode to deliver 12000 μ Jules, gave a higher signal to noise ratio than baking. In parallel experiments using RNA slot blots and a v-H-ras probe, the Nytran support membrane showed increased sensitivity when UV crosslinked, approximately 2 to 5 fold over baking. Another nylon membrane, Duralon (Stratagene) was also tested, but even though UV crosslinking gave a stronger signal, it was weaker than Nytran. The Southern blots were pre-hybridized in hybridization solution I (see appendix A) for 1 to 4 hours at 68°C and hybridized with a ³²P-labelled probe for human repeated sequences.

The BLUR8 plasmid containing a 300 bp human Alu family repeated sequence (41) was purchased from Amersham. The BamHI fragment containing human repeated sequences was electroeluted out of a new type of agarose called NUSIEVE (FMC Bioproducts, Rockland ME). This agarose separates DNA with resolution similar to polyacrylamide gels thus eliminating the toxic hazards associated with acrylamide. The BLUR8 insert probe was labelled by the random priming method of Feinberg and Vogelstein (1983) which employs random hexadeoxyribonucleotides to prime for DNA synthesis by the Klenow fragment of DNA polymerase and [α ³²P]dCTP (3000 Ci/mM, Amersham, Arlington Heights, IL). Routinely 30 to 40 ng of insert DNA was labelled for 2 hours at room temperature with 100 μ Ci [α ³²P]dCTP using the Pharmacia oligolabeling kit according to instructions provided by the manufacturer. The unincorporated nucleotides were removed by size exclusion chromatography on a prepacked Sephadex G-50 column (NICK column, Pharmacia) according to the instructions of the manufacturer. The buffer used for elution was 20 mM sodium phosphate pH 7.2, 0.5 mM EDTA, 0.05 % SDS. This type of column purification was

used for all DNA and oligonucleotide probe purifications. Hybridization conditions for Southern blots of genomic DNA with the BLUR8 probe were 5×10^6 cpm/ml probe in Hybridization Solution I at 65°C for 16 to 18 hours. The blots were washed once at room temperature for 5 minutes in 0.1 X SET, 0.1 % SDS, followed by 3 to 4 washes in the same buffer at 60°C for 20 minutes each. The blots were exposed to film (XAR5, Eastman Kodak, Rochester, NY) with intensifying screens (Lightning Plus, Dupont NEN, Boston MA) at -70°C overnight, followed by re-exposure for 3 to 5 days depending on the strength of the signal. Exposed films were developed using an automatic developer (Picker Intl.).

2. Screening for ras Genes

Primary tumors that were positive for human sequences using the BLUR8 probe were tested for the presence of ras sequences by hybridization with probes for H-, N- and K-ras. Probes for H-ras included a 700bp HindIII fragment from clone HI of v-H-ras (Ellis et al 1981) that contains most of the coding sequence of v-H-ras, and the 3.2kb SacI fragment from pT24c3 (Santos et al 1982, ATCC# 41000) including the entire active human c-H-ras oncogene without any repetitive sequences. Probes for various parts of the human N-ras gene were excised from the N-ras mini-gene plasmid, pNRSac (ATCC# 41031). Exon 1 was isolated on a 300bp HindIII fragment; exon 2, on a 760bp HindIII to EcoRI fragment; and exons 3 and 4 on a 4.5kb EcoRI to SacI fragment. Another probe specific for human N-ras 3' untranslated sequences was isolated on a 1.5kb PvuII fragment from p52c⁻ (Murray et al 1983, ATCC# 41030). Probes for the beginning and end of the c-K-ras gene were excised from a plasmid clone of human c-k-ras cDNA, pSW11-1 (McCoy et al 1984, ATCC# 41027). Exons 1, 2, and 3 were contained within a 480bp EcoRI to PstI fragment, and the end of exon 3 through 4b was contained on a 640bp EcoRI fragment. Another probe specific for human c-K-ras intronic sequences was isolated on a 640bp EcoRI to HindIII fragment from p640 (McCoy et al 1983, ATCC# 41026). This probe, like the probe from p52c⁻, does not cross-react with endogenous mouse c-k-ras sequences, and is useful for distinguishing

human genes from mouse genes. All ras probes were purified twice by sequential electrophoresis through agarose and electroelution to remove contaminating plasmid sequences, and were labelled by nick translation (Rigby et al 1977) using the BRL nick translation kit and 10 μ l (100 μ Ci)[α -³²P]dCTP (3000 Ci/mM, Amersham). Tumor DNAs were digested with EcoRI for detection of N-ras, K-ras and Alu sequences, and with BamHI for detection of human H-ras sequences, and separated in 0.8% agarose gels for Southern transfer to Nytran. Hybridization conditions were initially identical to those for the Alu probe. After some problems with background hybridization, the conditions were changed to a modification of the Church and Gilbert protocol (1984). The hybridization conditions were changed to Hybridization Solution II at 68°C for 15 to 18 hours followed by 3 washes in 2 mM Tris, pH 8.0, 2 mM EDTA, 0.1% SDS for 15 minutes each at 60 to 65°C.

D. Detection of RNA

1. Isolation of Total RNA from Tumor Tissue

Whole cell RNA was isolated from frozen tumor tissue by the method of Chomczynski and Sacchi (1987), with slight modifications. A small piece of tissue (less than 1 g) was thawed quickly and immediately homogenized with a sonicator in 5 ml solution D (see appendix C). The following reagents were added to the mixture with thorough mixing between each addition: 0.5 ml 2 M sodium acetate, pH 4, 0.5 ml water saturated phenol, and 1 ml chloroform: isoamyl alcohol (49:1). The final suspension was shaken vigorously and allowed to stand on ice for 15 minutes. The aqueous phase was separated by centrifugation at 10,000 g for 20 minutes at 4°C and the RNA was precipitated with an equal volume of isopropanol. The RNAs were precipitated at -20°C for at least 1 hour, usually overnight. The precipitate was pelleted in a microfuge for 20 min at 4°C and the pellet was redissolved in 0.5 ml Solution D. Rapid solubilization of the pellet was critical for good recovery of intact RNA; more Solution D was sometimes needed to accomplish this and each tube was treated individually. The RNA was then re-precipitated with 1 volume

isopropanol at -20°C for 1 hour and the precipitate was centrifuged as above. The pellet was washed 2X with 70% ethanol and dried under vacuum. The RNA was resuspended in 500 µl sterile RNase-free water, and a small aliquot was quantitated by OD at 260 using an extinction coefficient of 40 OD/µg RNA. The 260/280 ratio was routinely 1.9 to 2.0.

2. Isolation of Cytoplasmic RNA from Cells

Cytoplasmic RNA was isolated from tissue culture cells by a modification of the procedure of Favaloro et al (1980). All buffers are described in appendix C. Briefly, 1 to 2- T 175 flasks of cells were grown to 80 % confluence. The cells were trypsinized, washed once with medium containing serum to inactivate the trypsin and twice with cold DPBS. The final pellet was resuspended in 1 ml cold lysis buffer containing vanadyl ribonucleoside complexes to inhibit RNases and transferred to a polypropylene tube (2063 Falcon). The lysate was underlayered with 1 ml sucrose lysis buffer and allowed to stand on ice for 5 minutes. The nuclei were pelleted through the sucrose cushion at 6,000 g in a Sorvall RC5 centrifuge with an HS4 rotor at 5°C for 15 minutes. The upper cytoplasmic layer was transferred to a clean tube containing 1 volume of 2X PK buffer with 400 ug/ml proteinase K. The digestion was carried out at 56°C for 30 minutes. The RNA was extracted once with Phe:CHCl₃ and once with CHCl₃:IAA. The RNA was then precipitated with 2.5 volumes of 95 % ethanol at -20°C for 2 hours to overnight. The RNA precipitate was pelleted at 4,000 g in a Sorvall RC3 at 4°C for 30 minutes. The precipitate was washed with 80 % ethanol and dried under vacuum. RNA preparations were either re-extracted and precipitated with 0.3 M sodium acetate, pH 5.2, and ethanol, or used directly for poly-A⁺ RNA purification. Quantitation of cytoplasmic RNA was the same as for tumor RNA (see above).

3. Poly-A⁺ RNA Purification

Poly-A⁺ RNA was purified by oligo-dT cellulose column chromatography (Aviv and Leder 1972). Oligo-dT cellulose (12-18-mer, Pharmacia) was swollen in high salt buffer

(buffer formulas are in appendix C). The surry was allowed to settle and was resuspended in low salt buffer, allowed to settle again and resuspended in high salt buffer to pack the column. Small polypropylene columns (poly-prep columns, BioRad, CA) were siliconized (Maniatis et al 1982), DEPC treated (Kumar and Lindberg 1972) and stored in canisters until use. Columns were packed with 0.2 to 0.4 ml pre-swollen oligo-dT cellulose depending on amount of RNA to be processed. Typically 200 to 400 µg RNA were purified at a time to yield 5 to 10 µg poly-A⁺ RNA. The RNA was dissolved in low salt buffer and heated to 65°C for 5 minutes, then cooled quickly on ice to room temperature and NaCl was added to 0.5 M. The RNA was cycled through the column 5 times and the column was washed with 3 column volumes of high salt buffer. The poly-A⁺ RNA was eluted with at least 2.5 column volumes of low salt buffer and precipitated with 0.3 M sodium acetate, pH 5.5, and 2.5 volumes 95 % ethanol at -20°C overnight. The poly A⁺ RNA was stored precipitated until it was used for Northern blots or cDNA library construction.

4. Northern Blot Analysis

RNAs were separated in denaturing formaldehyde agarose gels and electroblotted to Nytran for hybridization. The recipe for formaldehyde agarose gels is in appendix C. Typically 15 µg of each total RNA sample was denatured in 20 µl loading buffer (see appendix C) at 65°C for 5 minutes and 4 µl of 5X STOP solution was added to facilitate loading. A marker lane was included in each gel (RNA ladder, BRL). The gels were run at 100 volts until the bromphenol blue dye had migrated through 3/4 of the gel. Since the concentration of ethidium bromide in the agarose was high, it was difficult to visualize the RNA directly in the gel. The gel was equilibrated in 1X TAE (see appendix A) and transferred to Nytran by electroblotting for 3 hours at 4°C in an IBI electroblotter at 25 volts, 800 mA according to the instructions of the manufacturer. The RNA bound to the Nytran support was then visualized with a Fotodyne transilluminator and pictures were taken of the blot. The RNA was fixed to the damp blot by UV crosslinking in the Stratalinker in the auto

crosslink mode. The blot was then air dried and pre-hybridized for at least 2 hours in hybridization buffer (see appendix C) at 55°C in a heat sealed bag. Blots were hybridized with the indicated probes at 55°C for 14 to 18 hours. Northern blots were washed with 0.1 X SET, 0.1 % SDS at 60 to 65°C at least 3 times until the background was low when scanned with a geiger counter. The damp Northern blots were wrapped in plastic wrap and exposed to film with intensifying screens as with Southern blots. The probe was stripped from Northern blots by incubation in 0.1 X SET, 0.1 % SDS, 50 % formamide at 80°C for 4 hours. The blots were rinsed in buffer without formamide and exposed to film to verify that the signal was gone. Stripped blots were kept moist and stored frozen at -20°C in sealed bags for re-hybridization.

E. Cloning and Sequencing the Transforming Genes

1. Genomic Library Construction and Screening (N-ras)

A genomic DNA library of EcoRI partially digested primary tumor DNA in the EMBL4 vector (Frishauf et al 1983) was screened for human N-ras containing clones. The library was prepared by digesting 6 pools of tumor DNA with a series of EcoRI concentrations from 0.75 U/μg to 0.12 U/μg for 30 minutes at 37°C in Universal restriction enzyme buffer. The partially digested DNAs were pooled and run slowly (30 mA) overnight in two large 0.6 % agarose gels without ethidium bromide. Molecular weight standards (high molecular weight ladder, BRL) were run in a side track and cut out for staining. The gel was aligned with the standards and the area equivalent to the 15 to 18 kb size range was cut out of the gel. The size-fractionated DNA was electro-eluted using 1 X TAE by placing the agarose gel slice into prepared dialysis tubing (3/4 inch, BRL) and purified on a NACS column (BRL) according to the instructions of the manufacturer. The eluted purified DNA was precipitated with 2 volumes of ethanol at -20°C overnight. Five μg Lambda EMBL4 (Frishauf et al 1983), purchased from Stratagene, was double digested to completion with excess EcoRI and

BamHI to prevent re-ligation of the stuffer sequence with the vector during construction of the library. The size fractionated DNA was ligated to the vector arms at two molecular ratios, 1:1 and 2:1 insert to vector (ie. 320 ng insert to 1 µg vector in a 10 µl reaction) with T4 DNA ligase (New England Biolabs) in 1 X ligase buffer (see appendix A) at 5°C overnight. The ligation products were packaged in phage heads using Gigapack goldTM packaging extracts (Stratagene). Briefly, 1 to 2 µl of the ligation mixture was mixed with 10 µl of the freeze-thaw extract and 15 µl of the sonic extract was immediately added. The packaging reaction was incubated at room temperature for 2 hours and stopped by the addition of 500 µl SM buffer and 20 µl chloroform.

Since the phage vector stuffer fragment carries the red and gam genes of the P2 phage, E.coli lysogenized with this phage will not support the growth of non-recombinant clones. The packaged library was titrated in a permissive and restrictive E.coli host, LE392 and P2392 respectively, to determine the percent recombinant phage. The phage titrations and large scale library screening were plated on LB agar plates with 100 µl infected cells in 2.5 ml top agarose for small plates and 300 µl infected cells in 6 ml for large plates. The phage were diluted in TM buffer and incubated with freshly prepared cells for 20 minutes to allow for attachment. The host cells were prepared by growing an overnight culture in LB broth plus 0.2 % maltose with shaking overnight at 37°C followed by centrifugation and resuspension of the pellet in 0.4 volumes of 10 mM MgSO₄. The prepared cells could be stored refrigerated for up to 5 days with only slightly decreased plating efficiency.

To screen the library, ten- 150mm petri dishes were seeded at approximately 20,000 recombinant phage/ plate and grown overnight. The next morning the plates were overlayed with nitrocellulose discs (HATF, Millipore) that had been numbered with permanent ink to correspond with the plate. Using a needle dipped in India ink the orientation of the discs were marked and the discs were carefully lifted off the plates and placed, phage side up, on a sheet of filter paper soaked with denaturing solution (see appendix A) for 3 minutes. The plates were overlayed with fresh nitrocellulose discs to make duplicate filters, and after 3 to

5 minutes they too were marked for orientation and placed in denaturing solution. All discs were treated as follows: two- 3 minute incubations in denaturing solution, and blotting to remove excess buffer, followed by two- 3 minute incubations in neutralization buffer and finally a brief submersion in 10 X SET. The filters were air dried and baked at 80°C under vacuum for 2 hours. The filters were sorted and prehybridized in two- 150 mm petri dishes at 68°C for 2 to 4 hours with 4 ml/ disc Hybridization buffer I. Since human N-ras is contained on two EcoRI fragments, and the library was by partial digestion with this enzyme, full length clones were picked by hybridizing one filter from each plate with a 3' probe and the other with a 5' probe. The filters were hybridized at 68°C for 12 hours and washed 4 times at 60°C in 0.1 X SET, 0.1 % SDS for 15 minutes each. The filters were air dried and exposed to film overnight. Positive spots from both hybridizations were aligned and double positive clones were picked for purification. Plugs of approximately 5 plaques were picked with the fat end of a glass pasteur pipet into 0.4 ml SM buffer. A drop of chloroform was added and the phage were allowed to diffuse into the buffer for 2 to 4 hours, then 25 µl of a 1:100 dilution was re-plated on permissive E.coli LE392. The phage were grown overnight again and re-probed as before. Well isolated positive plaques were picked with the small end of a pasteur pipet into 100 µl SM. 10 µl of these phage stocks were plated again to test for sufficient titer and for purity by a final hybridization with the 3' probe. Plate lysates of pure phage clones were grown for phage DNA preparation (see section on DNA). Southern blots of phage clone DNAs digested with EcoRI were hybridized with 3' and 5' probes and several full length clones were identified. Large scale phage DNA preparations were made for transfection into NIH 3T3 for tumorigenesis and focus forming assays, and for isolation of insert DNA to subclone.

The first subclone, pNe12, contained N-ras exons 1 and 2 on a 3.2 kb BglII to EcoRI fragment cloned into the plasmid vector pGEMblue (now renamed by Promega as pGEM5) double digested with BamHI and EcoRI. Exons 3 and 4 were subcloned as a 6.2 kb EcoRI fragment into the vector pGEM7z digested with EcoRI and dephosphorylated, named

pGNras3'Eco5. Further subclones from this plasmid were made for sequencing through the 3' untranslated exons: pN3'14 contains a 5 kb EcoRI to SacI fragment encompassing exon 3 through the beginning of exon 6, pN3'25 contains a 900 bp SacI fragment spanning a large portion of exon 6 (see diagram of N-ras sequencing strategy for location of sub-clones).

2. cDNA Library Construction and Screening (H-ras).

A cDNA library was constructed from reverse transcribed poly-A⁺ RNA from a nude mouse secondary tumor that contained large amounts of ras p21 without a correspondingly increased amount of ras mRNA, I921. The preparation of tumor RNA and poly-A⁺ RNA purification are described above. The cDNA library was prepared using the cDNA library synthesis kit from Pharmacia according to the directions of the manufacturer. Briefly, 5 µg of poly-A⁺ RNA, in RNase free water, was heated to 65°C for 5 minutes and reverse transcribed using oligo d(T)₁₂₋₁₈ with Moloney murine leukemia virus reverse transcriptase at 16°C for 1 hour. A trace amount of [$\alpha^{32}\text{P}$]dCTP (10 µCi) was added to the reaction as a means of evaluating the quality of the newly synthesized cDNA. The second strand synthesis reaction was done by a modification of the procedure of Gubler and Hoffman (1983) using RNase H and DNA polymerase I to replace the RNA with DNA by nick translation. The second strand reaction mix was incubated at 12°C for 1 hour followed by 22°C for 1 hour. The Klenow fragment was added and the incubation was continued for 30 minutes at 37°C to insure blunt ended cDNA. The cDNA was extracted once with Phe:CHCl₃ and a 5 µl aliquot was run in a 1% agarose gel and autoradiographed to check for adequate size distribution of the newly synthesized cDNA. The cDNA was purified away from unincorporated nucleotides by spin column chromatography on a Sephacryl S300 column equilibrated with ligase buffer (66 mM Tris, pH 7.6, 1 mM spermidine, 10 mM MgCl₂, 15 mM DTT, 44 µg/ml BSA). The effluent was collected and dephosphorylated EcoRI adaptors (supplied with the kit) were ligated to the cDNA by the addition of ATP and T4 DNA ligase and incubated overnight at 12°C. The ligase was inactivated by heating to

65°C for 10 minutes and the adaptor ligated cDNA was phosphorylated with T4 polynucleotide kinase and ATP at 37°C for 30 minutes. Another spin column was equilibrated with 20 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, and the kinase was inactivated by heating to 65°C for 10 minutes followed by extraction with Phe:ChCl₃. The cDNA was again purified by spin column chromatography and precipitated overnight at -20°C in ethanol. The precipitate was pelleted in a microfuge, washed with 80% ethanol and dried under vacuum. The cDNA was resuspended in water and ligated at two estimated molar ratios (1:1 and 2:1) to 1 µg of EcoRI digested, dephosphorylated lambda GT11 arms (Promega) for 14 hours at 12°C. The extent of ligation was checked by electrophoresing an aliquot of each reaction in a 0.7 % agarose minigel and looking for radioactivity in the high molecular weight region of the gel by autoradiography. The ligation reaction was packaged with Gigapack GoldTM (Stratagene) packaging extracts as with the genomic library. The resulting cDNA library was titrated in E.coli Y1090r⁻ on LB plates containing X-gal and IPTG in the top agar (see appendix B). This cloning vector contains an EcoRI cloning site within the lacZ gene (Young and Davis 1983), so when phage contain inserts that disrupt the coding sequence of beta galactosidase no functional enzyme is produced and the plaques are clear. The packaging efficiency and percent recombinant phage were determined by counting the total number of plaques and the number of blue non-recombinants.

The library was plated at 30,000 plaques per 150 mm plate on E.coli Y1090r⁻ without indicators, and lifted onto Colony/PlaqueScreen hybridization transfer membranes (DuPont NEN) according to the recommendations of the manufacture, and screened by hybridization with H-ras probes. These membranes bind DNA without baking or UV-crosslinking. The membranes were marked for orientation as with the previous library screening, but the processing of the membranes had to be modified. The lifted phage DNA was denatured by placing the membranes in individual 0.7 ml pools of 0.5 M NaOH on plastic wrap for 2 minutes. The excess buffer was blotted off with 3M paper and the membranes were re-treated with NaOH and blotted again. The membranes were neutralized with 1.0 M Tris, pH

7.5 two times with blotting in between, and dried at room temperature. The membranes were hybridized using Hybridization Solution II after a 2 hour pre-hybridization in the same buffer at 68°C. The library was first screened with 1×10^6 cpm/ml v-H-ras HindIII fragment probe. Plugs from positive areas of the plates were re-plated (as with the genomic library) and re-screened with the same probe and an oligonucleotide probe specific for the 3' untranslated region (UTR) of mouse c-H-ras, CB64 (see appendix F). The v-H-ras probe was labelled by nick-translation as described under probing for ras, and the oligonucleotide was end-labelled using [γ - 32 P]ATP and T4 polynucleotide kinase. 200 ng of purified oligonucleotide was incubated at 65°C for 5 minutes, and labelled in 70mM Tris, pH 7.5, 10 mM MgCl_2 , 5 mM DTT, 100 μCi [γ - 32 P]ATP, 10 U T4 polynucleotide kinase (BRL) in a final volume of 30 μl at 37°C for 30 minutes. The reaction was stopped by adding EDTA to 10 mM and TE buffer to 60 μl . The labelled oligonucleotide was purified by Sephadex G50 column chromatography on a pre-packed NICKTM column (Pharmacia). Membranes were hybridized with 2×10^6 cpm/ml in Hybridization solution III at 42°C for 12 hours, and washed 2 times at room temperature in 6 X SET, followed by 2 times at 55°C in 0.5 X SET, 0.1 % SDS and exposed to film overnight at -70°C with an intensifying screen. As with the genomic library, the positive clones were plaque purified twice and minipreparations of phage DNA were prepared from plate lysates using Lambdasorb.

The phage clone DNAs were digested with EcoRI and the clones with the largest inserts were subcloned into the EcoRI site of pGEM7zf⁺. The EcoRI cleaved phage inserts were cut out of agarose gels, electroeluted into dialysis bags (3/4 inch prepared dialysis tubing, BRL), extracted and precipitated with 0.5 volumes 7 M ammonium acetate and 2 volumes ethanol at -20°C overnight. The washed and resuspended insert DNAs were ligated to EcoRI cleaved and dephosphorylated vector DNA and the ligation products were transformed into E.coli DH5 α library efficiency competent cells (BRL) using the manufacturer's protocol for small volumes. Briefly, 1 μl of the ligation reaction was mixed with 50 μl competent cells on ice. The cells were incubated on ice for 30 minutes and heat

shocked at 42°C for 45 seconds. Ten volumes of room temperature SOC medium (BRL) were added and the cells were incubated for 1 hour at 37°C with shaking. The transformed cells were plated on LB + AMP plates containing Xgal to detect insert containing plasmid clones. Several white colonies were tested for the presence of the insert by cleavage of miniprep DNA with EcoRI and agarose gel electrophoresis followed by Southern blotting and hybridization with the v-H-ras probe used to screen the library. The orientation of the clones was determined by single and double digestions with selected restriction enzymes. Larger preparations of plasmid DNA were purified on Qiagen columns (see DNA purification) for sequencing.

3. Sequence Analysis (N-ras, H-ras)

All sequencing was done using double stranded plasmid DNA and [α -³⁵S]dATP by modifications of the dideoxy sequencing method of Sanger et al (1977). The coding exons of the N-ras genomic subclones were sequenced according to the strategy depicted in Fig.4 using oligonucleotides specific for the cloning vector RNA polymerase start sites and the sense and anti-sense strands of the insert DNA (see appendix F for table of sequencing oligonucleotides). The vector specific oligonucleotides S²⁶ and T7, were purchased from Promega. Exon 1 and 2 sequencing oligonucleotides were purchased from Clontech as part of a PCR amplification kit. Other sequence specific oligonucleotides were synthesized on a Biosearch Model 8700 Automatic Synthesizer using triester chemistries (kindly provided by Alvaro Puga, NICHD). All oligonucleotides were 20-mers with G + C contents as close to 50 % as possible. Oligonucleotides were received in concentrated ammonium hydroxide and were incubated at 56°C for 4 hours to cleave the protective blocking groups from the bases. The ammonium hydroxide was removed by lyophilization and the oligonucleotides were resuspended in 200 μ l water, and precipitated with 0.5 volumes 7 M ammonium acetate and 2 volumes ethanol at -20°C for 2 hours. The precipitates were pelleted in a microfuge, washed with 80 % ethanol, dried under vacuum, and resuspended

in 100 μ l water. The oligonucleotides were then purified over a NICK column (Pharmacia) equilibrated with TE buffer and collected in 400 μ l. The concentration of each purified oligonucleotide was crudely estimated using an extinction coefficient of 40 OD per mg ssDNA at 260nm. Oligonucleotides were diluted to 10 ng/ μ l for use in sequencing reactions.

Three sequencing kits were used. Originally reverse transcriptase and the Klenow fragment of DNA polymerase were used to sequence N-ras (GEM-SEQ K/RT sequencing kit, Promega). Then sequenase (Tabor and Richardson 1987), a modified version of T7 polymerase was shown to be a better general enzyme for sequencing and the sequencing kit from US Biochemical, Cleveland, OH, was used. To sequence through some regions of high G + C content, and to resolve some ambiguities, Taq polymerase (Thermal base sequencing kit, Stratagene) was used. The kits were used according to the instructions of the manufacturers for sequencing plasmid DNA with [α - 35 S]dATP. Plasmid DNA purified by Qiagen column chromatography (see DNA purification section) gave the best sequencing results. Plasmid DNA was denatured using essentially the same method (Zhang et al 1988) regardless of the enzyme used for sequencing; the only thing that differed was the quantity of DNA used per reaction. Briefly, plasmid DNA (2 μ g for The K/RT and Thermalbase systems, and 3 μ g for the Sequenase system) was suspended in 18 μ l water and incubated at 80°C for 5 minutes with 2 μ l 2 M NaOH, 2 mM EDTA. The denatured plasmid was neutralized with 3 μ l 3 M sodium acetate, pH 5.0 and precipitated with ethanol on dry ice for 15 minutes. The precipitate was pelleted in a microfuge, washed with 70 % ethanol and dried under vacuum.

When using the Gem-seq K/RT sequencing system the DNA was resuspended in 10 μ l buffer (10 mM Tris, pH 7.5, 50 mM NaCl for Klenow, and 34 mM Tris, pH 8.3, 50 mM NaCl, 5 mM MgCl₂, 5 mM DTT for AMV reverse transcriptase) containing 30 ng primer and incubated at 37°C for primer annealing. Primer extension and dideoxy-nucleotide incorporation were accomplished in one step as follows: 40 μ Ci [α - 35 S]dATP and 5 Units of the appropriate enzyme were added to the annealing mix and this mixture was immediately

divided, 3 µl each, among 4 tubes containing 3 µl each deoxy- ,dideoxy-nucleotide mixture (provided in the kit). The reactions were incubated for 20 minutes at 37°C for Klenow or 42°C for reverse transcriptase, 1 µl of chase solution was added to each tube and the incubation was continued for 15 minutes. The reactions were stopped by addition of 5 µl stop solution supplied with the kit and boiled for 3 minutes before loading into a sequencing gel.

The sequencing reactions using Sequenase required 3 µg of plasmid template but much less primer and [α -³⁵S]dATP. This system gave more reliable sequence data than the K/RT system and was used almost exclusively for sequencing the H-ras cDNA clones. The denatured plasmid was resuspended in 10 µl of 40 mM Tris, pH 7.5, 20 mM MgCl₂, 50 mM NaCl, 10 ng primer, heated to 65°C and allowed to cool slowly to room temperature for primer annealing. The primer was extended by adding 1 µl 1 M DTT, 2 µl labelling mix (1.5 µM each dGTP, dCTP, dTTP), 10 µCi [α -³⁵S]dATP, and 1 Unit Sequenase, and incubated at room temperature for 5 minutes. The primer extension mixture was then divided among 4 tubes containing 2.5 µl each of the deoxy- dideoxy chain termination mixes provided in the kit and incubated at 37°C for 5 minutes. The reactions were stopped by the addition of 4 µl stop solution provided and boiled for 2 minutes as above.

To resolve sequence ambiguities in regions of high G + C content, the Thermalbase Taq polymerase kit was used. Two micrograms of plasmid DNA was denatured for each primer as above. The plasmid was resuspended in 10 µl of 1 X Taq sequencing buffer (supplied as 10 X) containing 25 ng primer. The primer was extended by adding 2 µl sequencing extension mix, 20 µCi [α -³⁵S]dATP and 1 Unit Taq polymerase and incubating the mixture at 42°C for 10 minutes. The primer extension reaction was divided among 4 tubes containing 2 µl each of the deoxy- dideoxy nucleotide mixes supplied with the kit and incubated at 70°C for 5 minutes. The reaction was stopped by the addition of 5 µl stop solution provided and boiling for 2 minutes.

The BRL sequencing apparatus model S2 was used for all sequencing gels. The plates were treated as mentioned in appendix D and 0.8 % wedge gels were poured by placing a 2 inch long strip of 3 MM paper cut to the width of the spacer along the bottom of the gel before taping the plates together. Eight percent denaturing gels were prepared using SequagelTM premixed ingredients (see appendix D, purchased from National Diagnostics, Mannville NJ) on the night before sequencing. The sequencing gels were pre-run with 1 X TBE buffer (see appendix D) at 75 watts for approximately 45 minutes to warm up the system and to test the integrity of the wells produced by the Sharkstooth combs (fine, BRL). Approximately 3 μ l of each sequencing mix, G-A-T-C, was loaded in 4 adjacent wells and run at 75 watts until the bromphenol blue dye ran off the bottom and a second aliquot of the same reaction mixes was loaded in another set of 4 adjacent wells and run until the bromphenol blue dye ran off the bottom again. The gels were fixed with 10 % methanol, 10 % acetic acid and dried in a BioRad gel dryer at 80°C for 2 hours. The dried gels were exposed to film at -70°C for 24 to 48 hours.

The autoradiograms were read by hand and entered into a VAX 750 computer using the Wisconsin Genetics Computer Group sequence analysis software package and its fragment assembly system (Devereux et al 1984). All sequence analysis was performed using this software including searching for homologies with Genbank and setting up alignments between clones.

F. Analysis of mouse c-H-ras expression

1. PCR Amplification of RNA and DNA

To determine whether the alternative splicing detected by sequencing the cDNA clones was a general phenomenon in the expression of mouse c-H-ras, RNAs from several tumors, untransfected NIH 3T3 cells, and L cells were reverse transcribed with Moloney murine leukemia virus (MoMuLV) reverse transcriptase (BRL) and specific areas of the c-H-ras gene

were amplified with Taq polymerase using the polymerase chain reaction technique (Kawasaki, and Lang 1989). Briefly, 8 µg of either total RNA or cytoplasmic RNA was reverse transcribed from a primer specific for the 3' end of the mouse c-H-ras mRNA, CB74 (see appendix F for location) using the following conditions: 2.5 mM of each deoxynucleotide, 200 ng primer CB74, 100 µg/ml BSA, 50mM Tris, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 5 mM DTT, 80 U RNasin, 200 U MoMuLV reverse transcriptase in a 20 µl reaction volume at 37°C for 30 minutes. The reaction was boosted with an additional 100 units of reverse transcriptase and the incubation was continued for 30 minutes. The synthesis was stopped by the addition of 10mM EDTA and extraction with CHCl₃: IAA. The cDNA was precipitated with 0.5 volumes ammonium acetate and 2 volumes ethanol at -20°C. The cDNA precipitate was pelleted, washed with 70 % ethanol and dried under vacuum. The cDNA was resuspended in 8 µl sterile water and the equivalent of 1 µg input RNA was used to amplify specific regions of the c-H-ras message by the polymerase chain reaction.

The Perkin Elmer kit was used according to the instructions of the manufacturer at half of the normal reaction volume to save reagents. Using a reaction volume of 50µl, each 1 µg of cDNA was amplified in 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01 % gelatin, 0.2 mM each dNTP, 100 ng each primer, 1.2 U Taq polymerase overlaid with light mineral oil (Sigma). The Perkin Elmer Cetus DNA thermal cycler was set to execute the following program: 94°C, 30 seconds, followed by 30 cycles of 94°C, 1 minute, 55 °C, 2 minutes, 72°C, 2 minutes, with a final extension of the 72°C incubation to 7 minutes. The amplified DNA was stored at 4°C until further analysis. Positive control samples of both genomic DNA (1 µg/ reaction) and cloned cDNAs (10 ng plasmid/ reaction) were amplified at the same time using the same reaction conditions. The amplification products were analysed by 5 % polyacrylamide gel electrophoresis using the BRL vertical gel electrophoresis system, model V16-2, in 1 X TBE buffer at 200 volts until the bromophenol blue had migrated 75 % of the length of the gel. The gel was stained with 0.5 µg/ml ethidium bromide in 1 X TBE and

photographed. The gel was soaked in denaturing solution two times for 15 minutes each, rinsed with distilled water and transferred to neutralization solution for two- 15 minute washes and finally equilibrated with 0.5 X TBE. The amplified DNAs were then transferred to Nytran by electroblotting using 0.5 X TBE at 25 volts for 1 hour in a BioRad Transblot semi-dry transfer cell. The DNA was fixed to the membrane by UV-crosslinking and the blot was hybridized with a P³²-labelled oligo probe specific for the fragment being amplified (see above for oligonucleotide hybridization conditions).

2. Construction of Chimeric cDNA Clones

Plasmid subclones containing the 3 longest mouse c-H-ras cDNAs were used to make reciprocal constructs between the 3' UTRs, the coding sequence, and the beginning of the 5'UTRs. The original cDNA inserts were cloned into the EcoRI site of pGEM7zf⁺ oriented such that SP6 polymerase would synthesize sense RNA. The longest clone, pl6s, was full length from a predicted mRNA start site (Brown et al 1988) and including a 32 nucleotide long poly- A tract at the 3'end. This clone contained an un-spliced intron D in the 3'UTR. Compared with pl6s, pl76 was 55 bp shorter in the 5'UTR, and contained an alternately spliced intron D and a 40 base long poly-A tail at the 3'end. The final original cDNA subclone, pl10 was 35 bp shorter than pl6s in the 5'UTR, and intron D was spliced 'correctly', but intron C within the coding sequence was unspliced, and the clone ended with only 5 adenosines. Due to the unspliced intron B, pl1016 codes for a truncated protein. Reciprocal constructs were made between all three forms of the alternately spliced intron D. First, pras67 was constructed by replacing the NarI to SpeI fragment of pl6s, containing most of the coding sequence, with the corresponding fragment from pl76. During the construction of this plasmid, and all subclones cut with NarI, a 57 bp NarI fragment 10bp from the 5' end of the l6s cDNA was lost. The deleted fragment contains an upstream start codon that is normally out of frame for p21 translation. Plasmid, pras7, was constructed by replacing the NarI insert of pl6s with the NarI insert of pl76; this fragment contains the entire

coding sequence and most of the untranslated sequences except for the first 65 bp of the 5' end of the cDNA and the last 40 bp before the poly-A addition site and 32 A's. Similarly, pras10 was made by replacing the NarI insert of pl6s with the corresponding fragment from pl1019. The 3' end of pras7 was replaced with the 3' end of pras10 to change intron D to the correctly spliced configuration by double digestion with SpeI and BamHI and ligating the plasmid containing fragment of pras10 with the insert fragment of pras7. A similar construct, pras106, was made by ligating the BamHI to SpeI insert fragment from pl6s with the same plasmid fragment as above from pras10. pras1 was generated by digesting pl6s with NarI and religating to specifically delete the 57 bp NarI fragment from the 5'UTR.

3. In Vitro Transcription and RNase Protection Mapping

In vitro transcription of RNA probes or full length mRNA was done using purified viral polymerases specific for the SP6 and T7 promoter sequences found in the vector used to subclone the cDNAs, pGEM7zf⁺ (Promega). All subclones were in the same orientation with respect to the promoters, SP6 synthesized plus strand and T7 synthesized antisense RNA. Transcription and translation protocols were essentially the same as in the Promega Protocol and applications Guide. Briefly, high specific activity riboprobes were synthesized on linearized templates for use in protection experiments to finely map the clones in relation to each other. The subclone pl6s was digested to completion with BamHI (New England Biolabs) in Universal restriction buffer (appendix A) at 37°C for 1 hour. The extent of digestion was checked in a 1 % agarose minigel. EDTA was added to 10 mM to stop the reaction and proteinase K was added to 200ug/ml and the restriction enzyme and any extraneous RNases were digested for 30 minutes at 56°C. The linearized plasmid was then extracted with CHCl₃:IAA and the DNA was precipitated by the addition of 1/2 volume of 7M ammonium acetate and 2 volumes of 95 % ethanol on dry ice for 15 minutes. The precipitate was pelleted in a microfuge for 20 minutes and the pellet was washed with 80 % ethanol and dried under vacuum. 0.5ug of linearized plasmid was used for each RNA

synthesis reaction. Transcription buffers are available from Promega, and the [α - 32 P]CTP was purchased from New England Nuclear (NEG008H, 3000Ci/mM, 10mCi/ml) and used within 2 weeks. Using the following reaction conditions and nucleotide concentrations, the specific activity of the synthesized probes were 3×10^8 cpm/ μ g RNA: 40 mM Tris-HCl, pH 7.5, 6 mM MgCl_2 , 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.5 mM each of GTP, ATP and UTP, 12 μ M CTP and 50 μ Ci 32 P- α -CTP, 20 Units RNasin, and 15-20 Units of T7 polymerase in a final volume of 20 μ l. A parallel reaction using a mixture of control SP6 templates was run to use as a molecular weight marker for denaturing gel electrophoresis. The marker was made at a much lower specific activity by increasing the CTP concentration to 50 μ M and reducing the [α - 32 P]CTP to 10 μ Ci. The reactions were incubated at 37°C for 1 hour, then 1 Unit RQ1-DNase (Promega) per μ g template was added to digest the DNA template for 15 minutes at 37°C and stop the reaction. The probe was then diluted with an equal volume of RNase free 10mM Tris-HCl, pH 7.5, 10mM EDTA buffer and 100 ng yeast tRNA (type xs, SIGMA) was added as carrier, and the unincorporated nucleotides were removed by centrifugation through an RNA Quick Spin column (Boehringer Mannheim). The eluate was collected and 2 μ l were placed in a solvent free scintillation medium, Ready CapTM (Beckman), to be counted on the P 32 channel of a Beckman LS9000 scintillation counter. Approximately 2 ng of probe RNA (5×10^5 cpm) was used for each RNase protection experiment.

To determine whether the cDNA clones were identical in the coding sequences, the in vitro transcribed sense RNAs were hybridized with an antisense probe synthesized from pl6s-3 linearized with BamHI (see above). 20 ng of sense RNA was hybridized in solution with 2 ng (5×10^5 CPM) of probe antisense RNA in a total volume of 40 μ l. The hybridization buffer consisted of 80 % deionized formamide (see appendix C, FLUKA), 0.4 M NaCl, 40 mM PIPES, pH 6.8, 4 mM EDTA. Since the G + C content of H-ras is 57%, the hybridization mixture was heated to 80°C and allowed to cool to 55°C, which is the calculated melting temperature (T_m) - 25°C. The hybridization was incubated for 16 to 18 hours and the

mixture was diluted with 10 volumes of RNase buffer (10 mM Tris, pH 7.5, 0.3 M NaCl, 5 mM EDTA) and 40 U/ml RNase T1, 40 µg/ml RNase A were added to digest all probe sequences that were not complementary to the test RNA. The RNase digestion was incubated at 30°C for 30 minutes and was stopped by the addition of proteinase K to 200 µg/ml for a 15 minute incubation at 56°C. Carrier tRNA was added to the digested RNAs and the samples were extracted once with CHCl₃:IAA and precipitated with 2.5 volumes of ethanol on dry ice for 15 minutes. The RNA precipitates were pelleted in a microfuge for 20 minutes and the pellets were washed 3 times with 80% ethanol, 0.3 M sodium acetate to get rid of some of the digested nucleotides. The pellets were dried under vacuum and resuspended in 10 µl 25 mM Tris, pH 7.5. The protected fragments were resolved on a 5 % acrylamide-urea gel. A 5 µl aliquot was mixed with 2 X loading buffer (90 % formamide, 9 % glycerol, 2 X TBE, 0.04 % bromphenol blue), and the sample was either boiled for 3 minutes or heated to 80°C for 5 minutes before loading the gel. The gel was prepared using the same reagents as a sequencing gel except that a vertical gel electrophoresis system from BRL was used (Model V16-2), and the gel was run for 2 hours at 220 volts, until the bromphenol blue dye was at the edge of the gel. To visualize the fragments, the gel was dried in a BioRad gel drier at 80°C for 1 hour and exposed to X-ray film for 1 hour at room temperature. The sizes of the protected fragments were estimated relative to the migration of SP6 control RNAs (1.38kb, 550bases and 220 bases, positive control template for SP6 polymerase, Promega). The above procedure was performed with special precautions for handling radioactive materials.

4. Synthesis of Large Amounts of RNA for Translation

The reaction conditions for synthesis of large amounts of RNA were similar to that of probe synthesis except a higher concentration of linear template was used and all 4 nucleotides were at 0.5 mM in a final volume of 50 to 100 µl; yields of 5-10 µg RNA/µg template were routinely obtained. A typical synthesis reaction was performed on 2.5 µg of plasmid template that had been linearized at the XhoI site and prepared as above. Sense

strand RNA was synthesized with SP6 polymerase for 1 hour at 40°C. The template was digested as above but, instead of column purification, EDTA was added to 10 mM, and the reaction mixture was extracted with Phe:CHCl₃, re-extracted with CHCl₃:IAA, and precipitated with 0.3 volumes 7.5 M ammonium acetate and 2.5 volumes 95 % ethanol at -20°C for longer than 2 hours. The synthesized RNA was pelleted in a microfuge, washed with 70 % ethanol and dried under vacuum. The RNA was resuspended in 50 µl sterile RNase free water, and a small aliquot was quantitated by OD at 260 nm using an extinction coefficient of 40 OD/ug RNA. The 260/280 ratio was routinely 1.9 to 2.0.

In vitro RNAs were capped before translation using guanylyl transferase (BRL) in the presence of a reducing agent S-adenosyl-L-methionine (SAM, hydrogen sulfate, Boehringer Mannheim). Capping reaction conditions for 2 µg in vitro transcribed RNA were 50 mM Tris-HCl, pH 7.9, 1.25 mM MgCl₂, 6 mM KCl, 2.5 mM DTT, 100 µg/ml acetylated BSA (BRL), 30 U RNasin, 100 µM SAM, 40 µM GTP, and 1 U/µg guanylyl transferase, in a final volume of 60 µl at 37°C for 1 hour. The reaction was stopped by the addition of 10 mM EDTA and extraction with Phe:CHCl₃, followed by CHCl₃:IAA and precipitation with 0.3 M sodium acetate and 2.5 volumes ethanol at -20°C overnight. The capped RNA was washed and dried and resuspended in sterile RNase-free water for translation in rabbit reticulocyte lysates or injection into Xenopus oocytes. The intactness of the synthesized RNAs were determined by denaturing agarose electrophoresis (see Northern protocol).

G. Protein detection

1. Western Blot Analysis

When nude mouse tumors were positive for the presence of both a ras gene and its mRNA, extracts of tumor proteins were tested for the presence of ras p21 proteins by Western blotting. The tumor proteins were separated on 12 % SDS PAGE reducing gels, transferred to nitrocellulose (0.45 µ, Schleicher and Schuell) and ras p21s were visualized

with a monoclonal antibody (Futh et al 1982) that cross-reacts with H-, K-, and N-ras p21s (Oncogene Science, Manhasset, NY) and I¹²⁵ protein-A (affinity purified, Amersham).

Tissue proteins were extracted by the method of Srivastava (1985). Briefly, small pieces of tumor tissue (less than 0.5 g) were thawed quickly and placed into a small dounce homogenizer fitted with a ground glass plunger, and homogenized in 1 ml of protein extraction buffer (see appendix E). The homogenate was transferred to a sterile Eppendorf tube and centrifuged at 4°C for 20 minutes. The supernatant was stored immediately at -70°C until protein determinations were made.

Total protein concentration was measured using a commercially available BCA kit (Pierce, Rockford, IL) with dilutions of BSA (Albumin Standard, Pierce) as a standard. The BCA method is based on the standard biuret reaction and the Cu¹⁺ is detected by spectrophotometric measurement of the purple complex that is formed when it reacts with bicinchoninic acid. Briefly, the protein samples were diluted 1:5 in sterile water and 0.1 ml was mixed with 2.0 ml of the color reagent according to the standard protocol. After incubation for 30 minutes at 37°C, the OD₅₆₂ of the samples was read in a Beckman DU-7 spectrophotometer corrected to a blank of 1:5 diluted lysis buffer. Sample concentrations were extrapolated from a linear graph of the standard dilution series.

SDS polyacrylamide gel electrophoresis (SDS PAGE) of tumor proteins was done using the NOVEX precast mini-gel system according to the specifications of the manufacturer. Tumor protein extracts (40 ug/well) were diluted to 7 ul/well mixed with an equal volume of loading buffer (100 mM Tris pH 6.8, 4 % SDS, 20 % glycerol, 4.25 % β -mercaptoethanol, 0.1 % methylene blue), boiled for 3 minutes in a water bath, and loaded on duplicate 15 well-precast 12% SDS PAGE gels (NOVEX, Encinitas, CA). The gels were run in 1 X SDS PAGE buffer (see appendix E) at 35 mA per gel until the blue dye front was about to run off the bottom. The proteins were transferred to nitrocellulose using the BioRad Transblot cell electroblotting apparatus at 100 mA overnight at 5°C. See appendix E for the transfer buffer.

The Western blots were immediately blocked and p21 Ras was detected according to a protocol obtained from S. Srivastava (NCI). The blots were blocked in BLOTTO (see Appendix E) for 1 hour with rocking at room temperature. First antibodies, either normal rat serum for the negative control blot, or monoclonal antibody Y13-259 (Oncogene Science) were diluted 1:200 in fresh BLOTTO and added to the blocked blots in heat sealed bags to prevent evaporation. The antibodies were allowed to react for 2 1/2 to 3 hours at room temperature with rocking. The blots were then thoroughly washed in 3 changes of BLOTTO for 10 minutes each. The second antibody, rabbit anti-rat IgG (Accurate Chemicals, Westbury, N.Y.) was diluted 1:200 in BLOTTO and both filters were incubated back to back in the same bag for 1 hour at room temperature with rocking. During the second incubation, [¹²⁵I] protein-A (affinity purified, Amersham) was diluted to 2X10⁵ cpm in BLOTTO and non-specific binding was absorbed out by pre-incubation with a blocked blank nitrocellulose filter for at least 30 minutes. The blots were washed 3 times for 10 minutes each in BLOTTO and incubated with the pre-absorbed [¹²⁵I] protein-A for 20 to 30 minutes at room temperature in a sealed bag. After four- 10 minute washes in BLOTTO, the blots were rinsed in several changes of distilled water and exposed to film (XAR5, Kodak) overnight at -70°C. Several exposures were taken, from 7 hours to 5 days. The size of the detected ras proteins were calculated relative to pre-stained molecular weight markers (BRL).

2. In Vitro Translation.

To determine whether the differentially spliced c-H-ras clones were translated with different efficiencies, the in vitro transcribed mRNAs were translated in rabbit reticulocyte extracts and injected into Xenopus oocytes. Varying concentrations of capped SP6 transcribed mRNAs (500 ng, 200 ng, 100 ng) were translated in 50 µl reactions using Promega rabbit reticulocyte extracts according to the protocol of the manufacturer. A typical reaction was as follows: 100 ng capped mRNA in 1 µl water was added to a reticulocyte extract containing 35 µl lysate, 6 µl H₂O, 40 U RNasin, 1 µl 1mM amino acid

mixture minus methionine and 4 μ l [35 S]methionine (1200 Ci/mM, NEN, final [35 S]methionine concentration of 800 μ Ci/ml). The RNA was translated at 30°C and 6 μ l samples were taken at 15 minute intervals for 60 minutes. The reaction was stopped by pipetting the samples directly into an equal volume of protein extraction buffer and storing them on ice for quick analysis or frozen at -20°C for later analysis. Two controls were included in each experiment: a blank lysate containing no added RNA to rule out background bands and a positive control of Brome Mosaic Virus (BMV) RNA, included with the kit, that translates 5 viral proteins: 110, 97, 35, 20, and 15 kd. Translation products were separated in pre-cast 12 % SDS-PAGE gels as for tumor proteins except that the gels were fixed with 50 % methanol, 7 % acetic acid, soaked in Enlightening (Dupont), and the gels were dried in a BioRad Model 583 gel dryer at 60°C for 1 hour. The dried gels were exposed to X-ray film for varying lengths of time, from 2 hours to 2 days. The size of the translation products were determined relative to the BMV products and to 14 C molecular weight markers (BRL).

3. Immunoprecipitation.

To confirm that the translation products were ras proteins, aliquots of the 60 minute time points were immunoprecipitated with the anti-ras monoclonal antibody, Y13-259 as follows: 5 or 10 μ l lysate was mixed with 10 μ l monoclonal antibody (Oncogene Science) and 200 μ l protein extraction buffer (see appendix E) and incubated at 5°C for 1 hour. During this incubation, an aliquot of pre-swollen protein-A sepharose (CL-4B, Pharmacia) equivalent to 10 mg/reaction was washed two times with DPBS (DPBS without calcium and magnesium, Whittaker), and resuspended in 100 μ l DPBS/reaction. Since rat IgG does not bind well to protein-A, the sepharose beads were first coated with rabbit anti-rat IgG antibodies (Accurate) by incubation with 10 μ l antibody per 10 mg protein-A sepharose on a rotator at 4°C for 30 minutes. The coated sepharose beads were washed 3 times with wash buffer (see appendix E) and resuspended in 100 μ l wash buffer and added directly to the

first antibody reaction. The antibody coated protein-A sepharose was incubated with the first antibody reaction mix for 1 hour at 4°C on a rotator. The immunoprecipitate was then washed 3 times in wash buffer and a final time in wash buffer without detergents. The pellet was then resuspended in 2 X protein sample buffer and boiled for 3 minutes to release the ras protein and denature it. Samples were separated by 12 % SDS-PAGE using the NOVEX system as above. The gels were dehydrated in 50 % methanol 7 % acetic acid, soaked in Enlightening (Dupont) for 15 minutes and dried in a gel dryer (BioRad) at 60°C for 1 hour. The dried gels were exposed to film for varying times between 1 hour and 1 day at -70°C. The size of the immunoprecipitated proteins were estimated by migration relative to ¹⁴C-labelled protein molecular weight markers. Proteins translated in Xenopus oocytes were also immunoprecipitated as above, except that 300 µg of extracted oocyte proteins were incubated with the first antibody.

4. Translation in Xenopus Oocytes.

In vitro transcribed and capped mRNAs made from four different chimeric cDNA clones in pGEM7Zf⁺ were purified and adjusted to a concentration of 1 mg/ml in sterile water. The RNAs were injected into albino Xenopus laevis oocytes by Dr. Eric Ackerman (NCI) and incubated at 19°C for 4 hours to recover in Barth-Hepes saline (see appendix E). Approximately 20 oocytes were injected with each mRNA sample. Individual oocytes were observed for viability and transferred to separate wells since oocytes that touch each other during the labelling reaction may die. The buffer was removed and replaced with fresh buffer containing 200 µCi/ml [³⁵S]methionine, 20 µl/oocyte. The oocytes were incubated overnight at 19°C and any non-viable oocytes were discarded the next morning (survival was approximately 90 %). The oocytes were washed with a large volume of buffer without ³⁵S and those injected with the same sample were pooled and placed in a 1.5 ml eppendorf tube. The oocytes were allowed to settle to the bottom of the tube, the buffer was removed and the tube was immediately placed on dry ice. When all pools were processed, the

oocytes were thawed and homogenized with 5 μ l protein extraction buffer per oocyte using a small glass rod. The tubes were centrifuged at 4°C in a microfuge for 15 minutes. Three layers were visible, an upper lipid layer, a middle aqueous layer and a lower yolk layer. The solubilized protein extract (middle) was removed using a thin tapered pipet tip. The protein concentration was measured using the BCA kit as above, and 300 μ g of each protein extract was immunoprecipitated to visualize the translated ras protein. A pool of uninjected oocytes served as a negative control.

Chapter III

RESULTS

A. Tumorigenesis Assay rationale and early experiments

Activated oncogenes have been isolated from the DNA of many human tumors and tumor cell lines by induction of focus formation in NIH 3T3 cells (for review see Bishop 1987). The classic focus formation assay has the limitation that the transfected oncogene must cause distinct morphological changes in the recipient cell for detection. More recently, tumorigenesis assays have been developed that have the potential to detect oncogenes which cause more subtle changes in cellular morphology but induce tumorigenesis (Keath et al 1984). To separate any potential transforming genes from the original tumor DNA, should more than one be activated, a sib-selection step was included in a modification of the co-transfection tumorigenesis assay described by Fasano et al (1984). The general scheme is represented in Fig.1. The assay consists of co-transfection of donor tumor DNA with pSV2neo and separation of the G418 resistant colonies into unique pools of 300 to 500 colonies each for injection into nude mice. It has been shown that cells taking up co-transfected DNA will on the average incorporate the equivalent of 0.1 % of the donor DNA genome (Wigler et al 1978). To have a 99% chance of detecting an oncogene if it is single copy in the donor genome, approximately 4,000 to 5,000 individual colonies must be screened, as calculated from the Poisson distribution equation $N = \ln(1-P) / \ln(1-f)$, where P is the desired probability, f is the fraction of the genome represented by each colony (in this case 0.001), and N is the number of colonies that need to be screened (Clarke and Carbon 1976). To use the least possible number of mice and still have a high probability of separating any potential oncogenes into different primary tumors, 10 pools of 300 to 500 colonies each were used for injection into 10 groups of 4 to 5 mice each. Mice were

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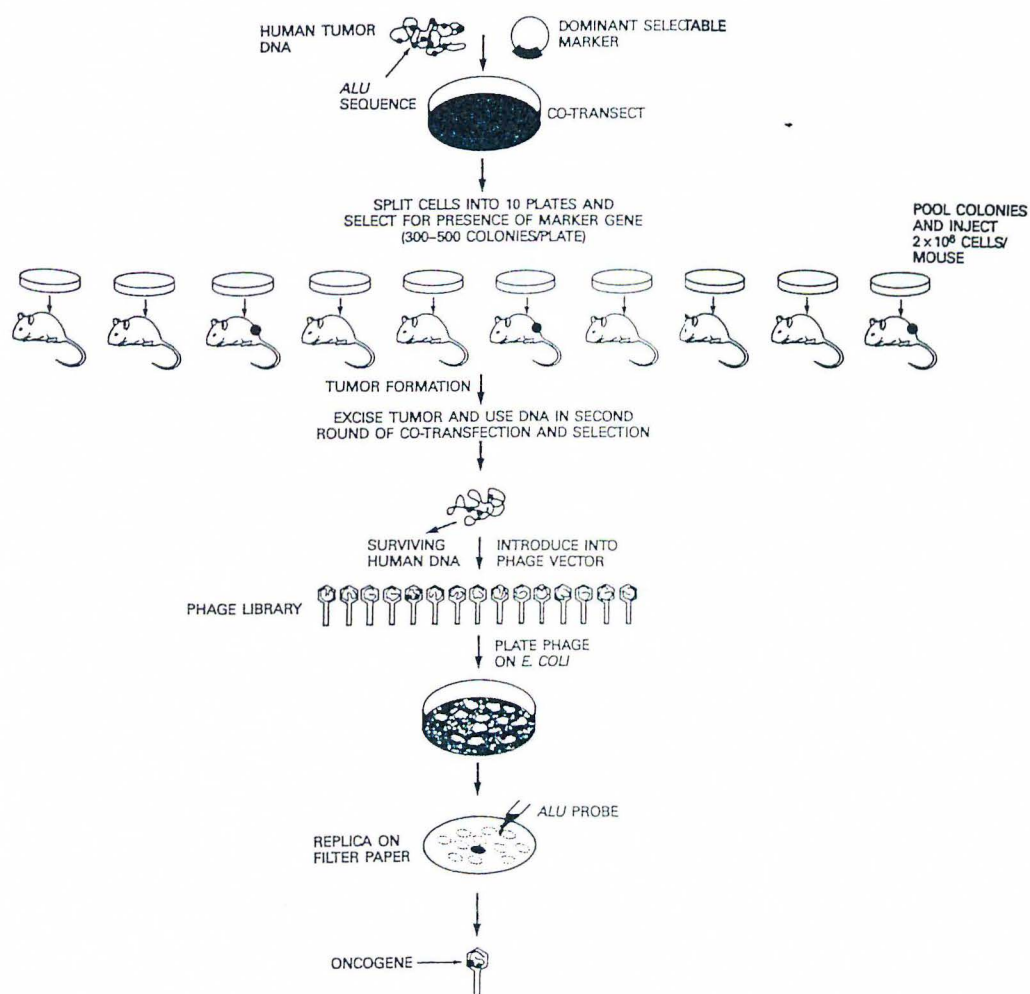


Figure 1. Schematic diagram of the sib-selection tumorigenesis assay to detect and isolate human oncogenes.

observed weekly for tumor formation at the site of subcutaneous inoculation for 15 to 16 weeks. DNA from the T24 bladder carcinoma cell line produced an average transfection efficiency of 0.13 foci/ μ g in focus assays and 5/5 tumors within 2 to 3 weeks in tumorigenesis assays. Negative controls included pSV2neo co-transfected with NIH 3T3 cell carrier DNA, a salt control consisting of the calcium phosphate precipitate without any DNA and a medium control that did not receive any treatment during the transfection. The spontaneous transformation rate of the NIH 3T3 cells was low, with sporadic formation of tumors after 14 to 15 weeks. Representative tumors from any pool that generated tumors in more than half of the mice injected were analysed for the presence of human repetitive sequences, indicative of the presence of a transfected human oncogene. Primary tumor DNAs containing human sequences were used in a second round of co-transfection and tumorigenesis to separate the potential oncogene from any un-related co-transfected human sequences. The secondary tumors were again tested for the presence of human repeated sequences.

To detect potential transfected human oncogenes in nude mouse tumor DNAs, Southern blots were first hybridized with a probe for the presence of integrated human DNA, as shown in Fig.2. The BLUR8 probe (Jelinek et al 1980) for the Alu family of human repetitive sequences was used for detecting human DNA. The Alu family sequence motifs are highly repeated throughout the human genome, with a random interspersion frequency of several kilobases. Since, on the average, a transfected cell incorporates 0.01 percent of the genome of the donor DNA, this repeat family should be well represented in the primary tumor DNAs. The BLUR8 probe hybridized to many fragments in Southern blots of primary nude mouse tumor DNAs (Fig.2, lanes G54, G55), to a few specific fragments of human DNA in positive secondary tumor DNAs (lanes G54-1, G54-F), and did not hybridize with untransfected NIH 3T3 cell DNA or DNA from spontaneous transformants that did not integrate human sequences (lane G55-4). Since activated ras genes have been isolated

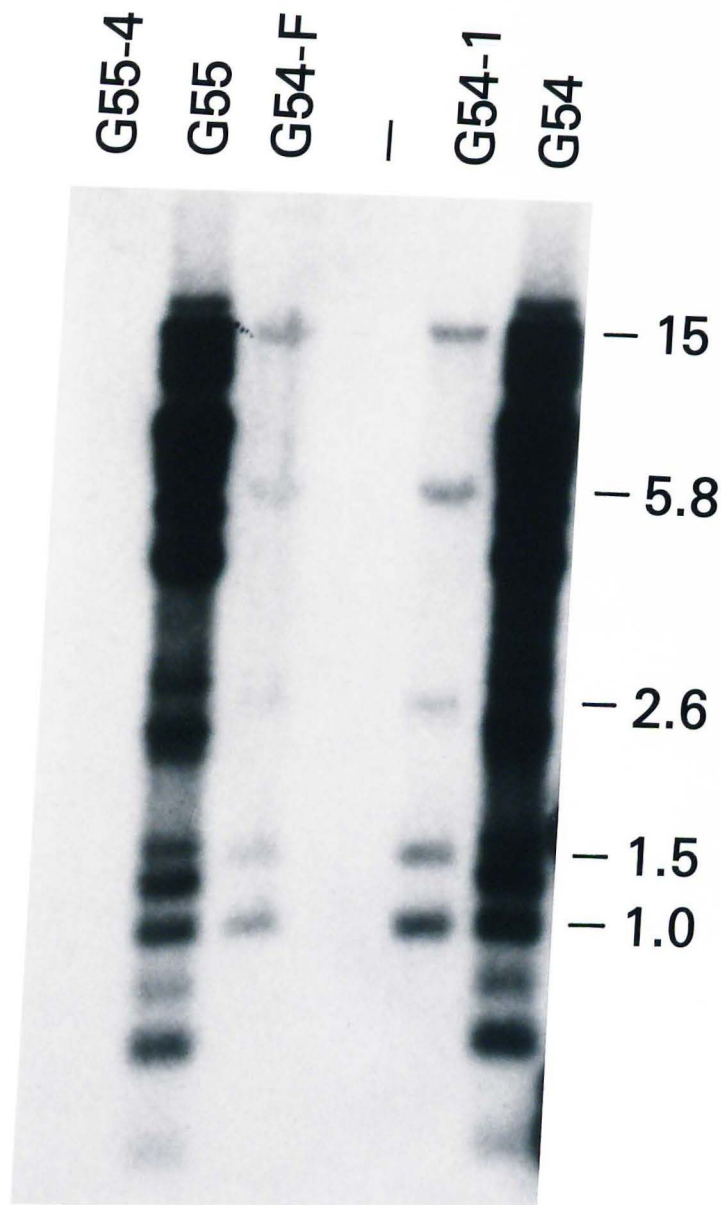


Figure 2. **Southern blot analysis of primary and secondary nude mouse tumor DNAs for human repetitive sequences.** Tumor DNAs (10 μ g) were digested with *Eco*RI, separated by electrophoresis in 0.8% agarose gel, blotted, and hybridized with the BLUR8 probe for human Alu repetitive sequences. Lanes G54, G55 are primary tumor DNAs; G54-1, G54-F are DNAs from a secondary tumor and pooled foci induced by G54 DNA; G55-4 is a spontaneous transformant derived NIH 3T3 cell tumor. Sizes (in kilobasepairs) of the Alu positive fragments in the secondary transfectant DNAs are indicated to the right.

most often from human tumors, all nude mouse tumor DNAs positive for human sequences were also screened for the presence of ras sequences with H-ras, K-ras and N-ras probes.

Insulinoma and renal cell carcinoma are two tumor types that have been associated with chromosomal changes indicative of the loss of specific tumor suppressor genes (Larsson et al 1988, Zbar et al 1987). To determine whether any dominant acting oncogenes were also involved in the genesis of these tumor types, representative tumors were screened by the sib-selection assay. Two renal cell carcinoma (RCC) DNAs were screened both by the tumorigenesis assay, and for focus formation on NIH 3T3 cells. Both DNAs were negative for focus formation, but one induced Alu positive nude mouse tumors and was further characterized. Of three insulinoma DNAs tested, all were negative for focus formation in primary transfection experiments, but induced primary tumors in the nude mouse tumorigenesis assay. One induced tumors through a third round of transfection and tumorigenesis and was chosen for further characterization. Although no novel dominant acting oncogenes were found in these tumor types, the increased sensitivity of the tumorigenesis assay allowed the detection of over-expressed ras proto-oncogenes activated during transfection. The mechanisms by which ras proto-oncogenes may be overexpressed and cause NIH 3T3 cells to become tumorigenic is the subject of this thesis.

B. Renal Cell Carcinoma

1. Detection of Human N-ras in Mouse Tumors

DNAs from two renal cell carcinomas (K and L) were obtained from Dr. Marsden Linehan (NCI) and were tested for the presence of activated oncogenes by the sib-selection tumorigenesis assay. One pool out of 10 induced the development of Alu positive primary tumors from tumor-K DNA in four out of five mice within 9 weeks of injection; two out of five mice injected with a second pool of primary transformants developed Alu negative tumors at 12 and 14 weeks after injection, probably due to spontaneous transformants. The rest of the

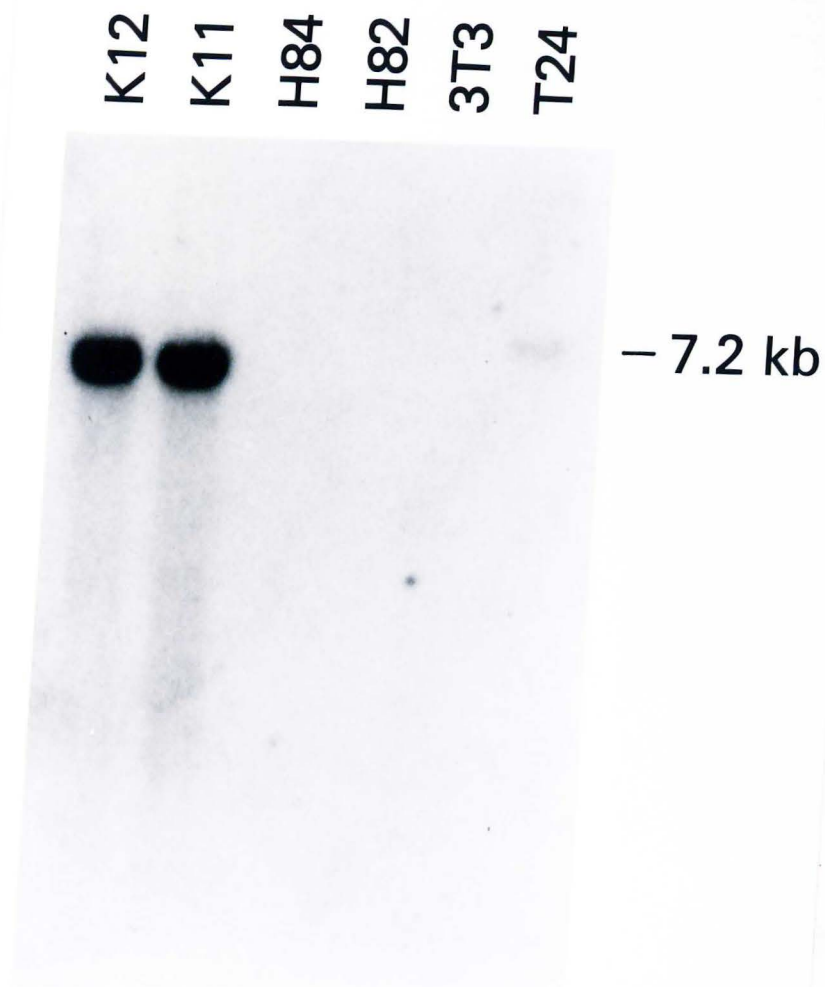


Figure 3. **Southern blot analysis of primary tumor DNAs for human N-ras.** Genomic DNAs from primary tumors K12, K11, H84, H82, untransfected NIH 3T3 cells and T24 human bladder carcinoma cells were digested with EcoRI, separated by electrophoresis through 0.8% agarose (10µg/lane), blotted, and hybridized with a human N-ras specific probe, p52c⁻. The 7.2 kilobasepair human N-ras fragment is indicated.

pools transfected with K-DNA did not induce tumor formation. Renal cell carcinoma-L DNA was negative in the tumorigenesis assay. To determine whether an activated ras oncogene was associated with the observed tumorigenesis, Southern blots of Alu positive primary nude mouse tumor DNAs were hybridized with ras specific probes. The human N-ras specific probe from p52c⁻ (Murray et al 1983) hybridized with a 7 kb EcoRI fragment in both Alu positive primary nude mouse tumor DNAs tested and did not hybridize with untransfected NIH 3T3 cell DNA or DNA from other primary tumors (Fig.3). By comparison with the single copy N-ras gene in human T24 bladder carcinoma DNA, the primary nude mouse tumor DNAs appeared to contain approximately 50 copies of N-ras. One of these primary tumor DNAs induced Alu positive, N-ras positive nude mouse tumors in a secondary co-transfection and tumorigenesis assay with higher incidence (all mice injected developed tumors) and shorter latency (5 weeks). To determine whether activation of the N-ras gene resulted from a point mutation, as had recently been detected by Bos et al (1985) using a similar assay, a genomic library was made from one of the primary tumor DNAs to clone and sequence the transfected oncogene.

2. Cloning and Sequencing the N-ras Gene

The active N-ras gene consists of 6 exons distributed over two EcoRI fragments (Hall and Brown 1985) (Fig.4). A genomic library of EcoRI partially digested, size fractionated primary tumor DNA was produced using the lambda vector, EMBL4 (Frishauf et al 1983). A library of 5×10^5 independent phage clones was generated that contained approximately 90% recombinants. Since the gene was highly amplified in the DNA used to make the library, approximately 5 to 10 positive plaques were detected per plate of 20,000 recombinant phage. Six full length clones were isolated by double screening the library with probes specific for both halves of the human N-ras gene. Four of the full length clones were tested in the tumorigenesis assay, using a codon 61 mutant (Bos et al 1984) N-ras minigene plasmid, pNRSac, as positive control, and a clone isolated from the nude mouse tumor

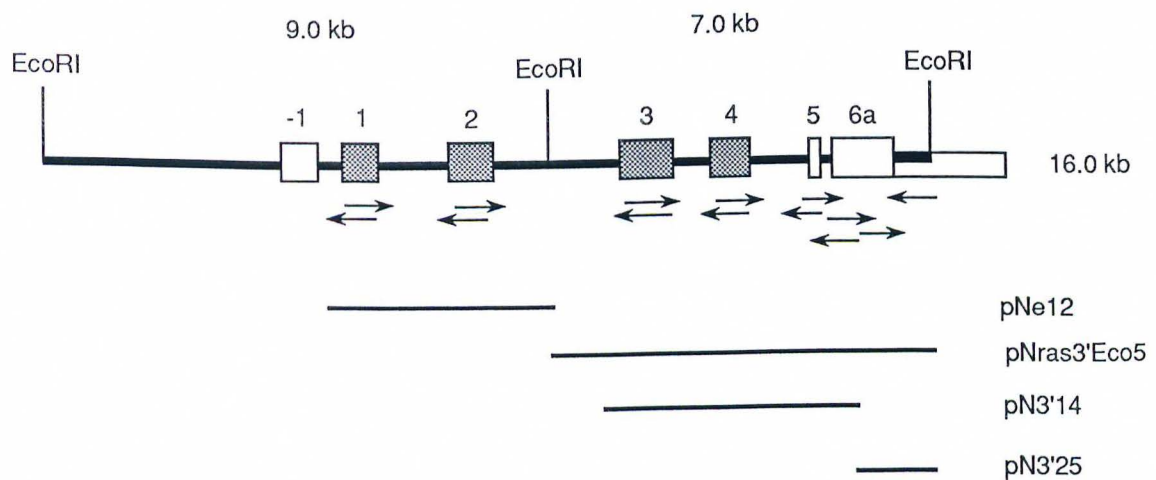


Figure 4. **Human N-ras genomic structure and sequencing scheme.** The coding exons are represented by shaded boxes, the non-coding exons are represented by open boxes, the introns and surrounding sequences are indicated by black lines. The recognition sites for *EcoRI* are indicated above the diagram. The regions of the gene subcloned for sequencing are indicated by solid lines with the names of the subclones to the right. The arrows below the diagram indicate the direction and extent of sequencing.

library that contained only the 5' half of the N-ras gene, λ ex1, as a negative control. The results of this experiment are summarized in Table 1. All four full length N-ras clones efficiently induced tumorigenesis, but with a slightly longer latency period as compared with pNRSac. These phage clones were also tested for focus formation but failed to form distinguishable foci in 3 weeks while the positive control, pNRSac, produced approximately 100 easily distinguishable foci/ μ g.

Since N-ras with codon 13 mutations had recently been shown to induce tumors in nude mice while causing very little alteration in NIH 3T3 cell morphology (Bos et al 1985), the possibility that this or a similar mutation had activated the gene was pursued by subcloning regions of the cloned N-ras gene and sequencing the coding exons. The location of the subclones and the sequencing strategy are depicted in Fig.4. Since the sequence of human N-ras was known, oligonucleotides specific for the first two exons were obtained commercially, and additional sequence specific primers were synthesized to directly sequence the coding exons (see Appendix F for primer sequences and locations). No point mutations were found in the coding sequence of the N-ras gene, though a few base changes and a small deletion were noted in the 3' untranslated region. Since ras genes can cause transformation either by point mutations in any of three known regions of the gene, or by over-expression of the normal protein (reviewed in Sistonen and Alitalo 1986) the possibility of an alteration in regulatory sequences or other untranslated regions causing tumorigenesis was considered.

3. Activation of N-ras by Gene Amplification

The standard method to localize which part of a gene contains an activating mutation is to test reciprocal constructs between the different parts of the normal and mutant gene for the ability to confer a detectable phenotype to indicator cells. To first determine whether transfection of the normal c-N-ras gene was negative in the tumorigenesis assay, I obtained a lambda clone of human c-N-ras (Yuasa et al 1984) from Dr. Stuart Aaronson (NCI) and

TABLE 1. Tumorigeneis Analysis of N-ras Lambda Phage Clones

TRANSFECTED DNA	TUMOR FORMATION		RAS DETECTION	
	Incidence	Onset ^a (weeks)	DNA ^b	Protein ^c
phage clones of <u>N-ras</u> (500ng/dish)				
λ N21	5/5	4	+	+
λ NE	5/5	4	+	+
λ N23	5/5	4	nd	nd ^d
λ N14	5/5	4	nd	nd
λ ex1 (neg. cont.)	0/5			
pNRsac (pos. cont.)	5/5	2	nd	nd
<u>N-ras</u> clone vs. <u>c-N-ras</u> (250ng/dish)				
λ N21	5/5	5	+	+
P798 (<u>c-N-ras</u>)	5/5	6	+	+

- a Time between injection of mice and detection of first palpable tumor. All mice within a group developed tumors within one week of the time of onset.
- b Detection of N-ras specific sequences on genomic DNA of the induced tumors by Southern blot analysis.
- c Detection of p21 Ras by Western blot analysis
- d nd = Not determined.

tested it in parallel with one of the full length clones isolated from the nude mouse tumor library. Surprisingly, DNA from the normal c-N-ras gene induced tumorigenesis with similar incidence and onset as compared with the gene cloned from the mouse tumor (Table 1). The first phage clone transfection and tumorigenesis experiment was done using 500 ng phage DNA/ plate; the second transfection was performed using half as much DNA, resulting in tumor induction with a longer latency.

To determine whether N-ras p21 was expressed in the nude mouse tumors, Western blots of tumor proteins were prepared. A combination of Y13-259, a monoclonal antibody that cross-reacts with all ras p21's, and ¹²⁵I-labeled protein-A coated with rabbit anti-rat antibodies was used to visualize the ras protein. All K-series nude mouse tumors tested by Western blot analysis showed dramatic overexpression of p21 Ras protein, including the tumors induced by normal N-ras. As shown in Fig.5, p21 Ras was detected in the N-ras induced tumors, including a secondary nude mouse tumor induced by K11 DNA, tumors induced by the full length N-ras clone λ N21, and two tumors induced by the normal c-N-ras clone, λ cNras; no p21 was detected in NIH 3T3 cell protein extracts or in a spontaneous tumor arising from a pSV2neo transfectant. Western blot results of phage clone induced tumors are summarized in Table 1.

Southern blot hybridization of genomic DNAs from the tumors induced by both control and test N-ras phage clones showed high copy numbers of integrated N-ras, as had been previously detected in the primary and secondary nude mouse tumor DNAs. However, blot hybridization of the original human tumor DNA and three metastases from the same patient showed that the N-ras gene was not amplified in the patient tumor tissues (Fig.6). It was concluded from these experiments that an amplification event had occurred during the primary transfection, and that integration and expression of amplified copies of transfected normal N-ras is sufficient to produce tumors in nude mice.

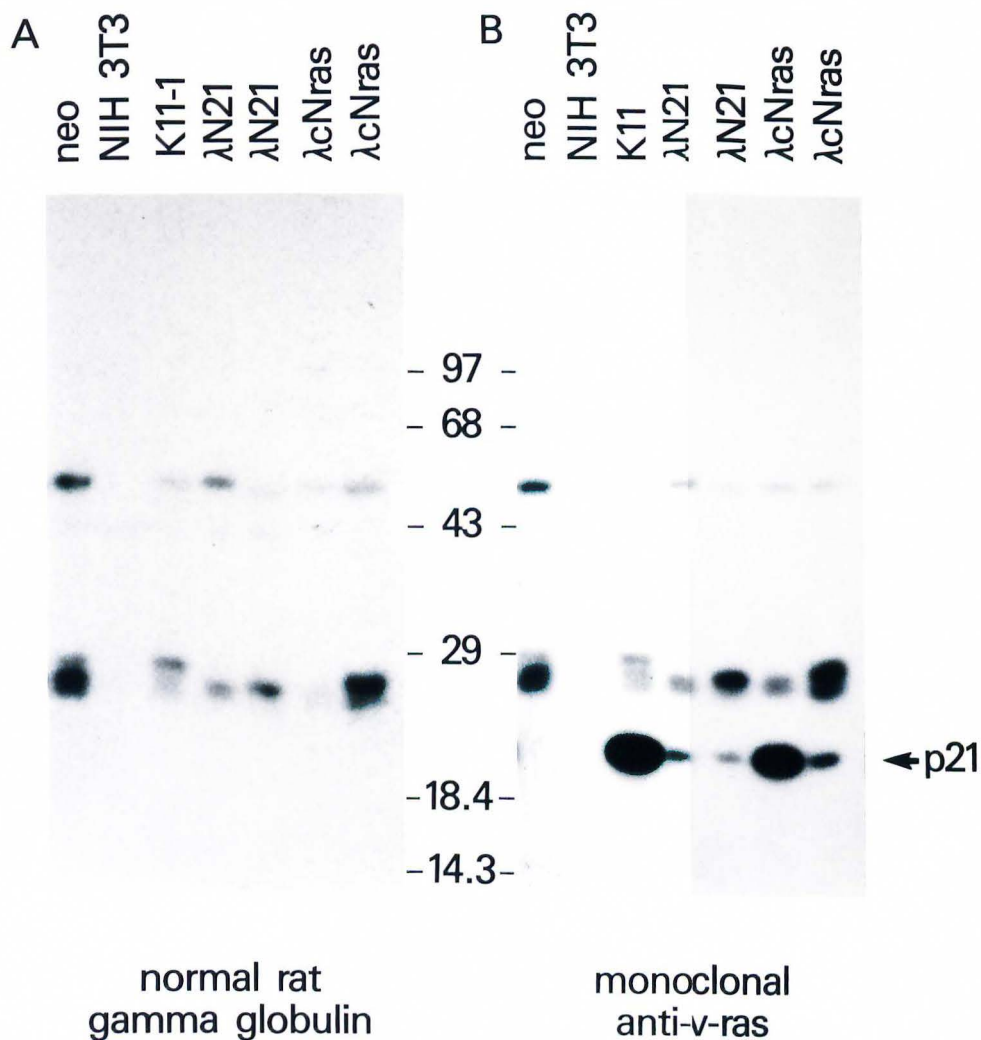


Figure 5. **Western blot analysis of N-ras induced nude mouse tumors.** 30 µg of protein extracts from tumors induced by transfection of human proto-oncogene c-N-ras, λcNras; a phage clone isolated from the K12 genomic library, λN21; a secondary tumor induced by K11 DNA, K11-1 (labelled K11 in panel B); a spontaneous tumor derived from transfection with pSV2neo, neo; and untransfected NIH 3T3 cells, NIH 3T3, were separated in a 12 % SDS polyacrylamide gel, and blotted. Panel A, first antibody was normal rat gamma globulin. Panel B, first antibody was Y13-259 anti-ras monoclonal antibody. Antibody binding was visualized by [¹²⁵I]protein-A and rabbit anti-rat antibody followed by fluorography. The relative migration of pre-stained molecular weight markers is indicated (X1000) and p21 Ras is indicated by the arrow on the right.

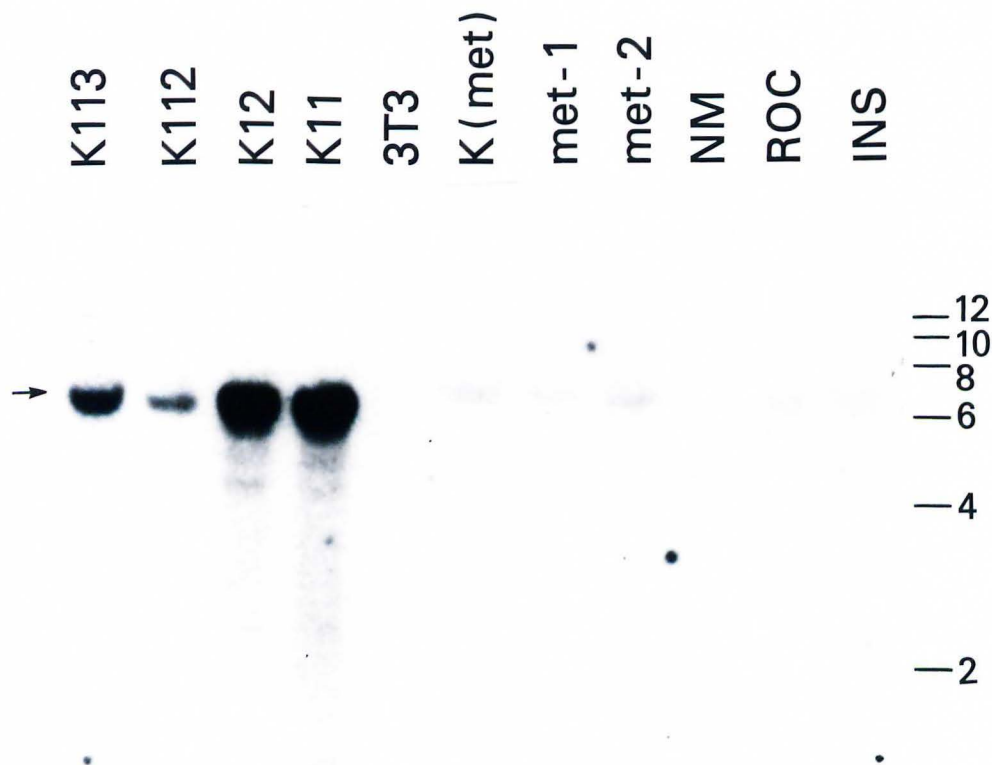


Figure 6. **Southern blot analysis of human, and nude mouse tumor DNAs for the presence of human *N-ras* sequences.** All DNAs were digested with *Eco*RI and 10 μ g/lane were separated in 0.8 % agarose, blotted and hybridized with the human *N-ras* specific probe from p52c⁻. DNA samples were from secondary nude mouse tumors, K113, K112; primary nude mouse tumors, K12, K11; untransfected NIH 3T3 cells, 3T3; human renal cell carcinoma (RCC) lymph nodal metastasis used to induce the K series tumors, K(met); two other metastases from the same patient, met-1, met-2; the patient's primary tumor propagated in nude mice, NM (this sample is contaminated with mouse cells and represents less than single copy human sequences); the primary tumor, ROC; and insulinoma I, INS. The relative migration of selected fragments from the 1 kb ladder (BRL) are indicated in kilobasepairs at the right. The arrow indicates the human *N-ras* specific fragment.

C. Insulinoma

1. Screening Human Insulinoma DNAs for Activated Oncogenes

Three human insulinoma DNAs were screened for active oncogenes by the sib-selection tumorigenesis assay. The results of these assays are summarized in Table 2. All insulinoma DNAs induced tumor formation in primary transfections. Primary pools of transfected NIH 3T3 cells were designated by a two part code, the letter represents the donor tumor DNA and the number designates which of the ten pools of cells were used for injection. The primary tumors were then named by this two part code followed by the number of the mouse that was injected, usually 1 through 5. A third number was added to the code used to name secondary tumors arising from transfection of the primary tumor DNA, designating again which mouse of the group injected bore the tumor. For example, G54 is a primary tumor from the fourth mouse injected with pool-G5 cells, and G54-1 is a secondary tumor generated by transfection of G54 tumor DNA.

Of ten pools co-transfected with Insulinoma-G DNA, two induced Alu positive primary nude mouse tumors. A third pool, G3, induced Alu negative tumors at a lower frequency and later onset, indicative of spontaneous transformation. Genomic DNA from the Alu positive primary tumor G54 induced Alu positive secondary tumors in 3/5 mice over a period of 5 to 10 weeks in one of three transfection and tumorigenesis experiments. In the one positive secondary transfection experiment, a parallel pool of G54 transfected NIH 3T3 cells was positive for focus formation, designated G54-F. DNA from both a secondary tumor, and the pooled foci exhibited the same pattern of Alu fragments in Southern blots hybridized with the BLUR8 probe (Fig.2, lanes G54-F and G54-1). Genomic DNAs from both G54-1 and G54-F, however, failed to induce tertiary tumors in four separate experiments. This could be due to either the presence of a very large oncogene that is difficult to transfect intact or that the human sequences were adventitiously transferred to secondary tumors in the one positive experiment.

TABLE 2. Tumorigenesis Analysis of Human Insulinoma DNAs

POOL ^a	TUMOR FORMATION		HYBRIDIZATION	
	Incidence	Onset ^b (weeks)	Alu	<u>ras</u>
INSULINOMA G				
G3	2/5	15	-	-
G4	3/5	13	+	-
G5	5/5	10	+	-
INSULINOMA H				
H4	2/4	11-14	+	-
H8	3/4	11	+	+
H10	2/4	8-14	+	-
INSULINOMA I				
I2	5/5	7-9	+	+
I6	5/5	9-11	+	+
I9	5/5	5	+	-
CONTROLS				
T24 <u>ras</u>	10/10	3	+	+
pSV2neo ^c	4/38	12-15	-	-

- a Only pools inducing 2 or more tumors are shown. All assays were done with 10 pools.
- b Time between injection of mice with transfected NIH 3T3 cells and detection of first palpable tumor. In most cases, all mice within a group developed tumors within one week of the time of onset. If not, a range is given representing time of onset of the first and last tumor.
- c Summary of relative tumor incidence by spontaneous transformation of NIH 3T3 cells co-transfected with pSV2neo and NIH 3T3 cell carrier DNA in 10 separate transfections.

Insulinoma-H DNA induced Alu positive primary tumors in 3 out of 10 pools with low incidence and varying latencies (Table 2). None of these tumor DNAs induced secondary nude mouse tumors upon repeated transfections. In ten secondary transfection and tumorigenesis assays, only two mice developed tumors and they were Alu negative. Insulinoma-I DNA induced Alu positive tumors in 3 out of 10 pools from the primary transfection. Three Alu positive primary tumor DNAs from pool-I2 hybridized with the v-K-ras probe but failed to induce Alu or ras positive secondary tumors in three separate transfections (Table 3). Genomic DNA from primary tumor, I92, produced both secondary, and tertiary tumors and was chosen for further analysis.

2. Analysis of the I-9 Series Nude Mouse Tumors

Representative tumors from each pool of transfected NIH 3T3 cells generating two or more tumors were screened for the presence of human DNA and activated ras oncogenes by Southern blot hybridization. Southern blot analysis was done with both Alu and ras probes to primary, secondary, and tertiary I-9 series nude mouse tumor DNAs. The code used to designate the origin of the I-9 series of nude mouse tumors was the same as that for the G and H tumors with one exception. When two separate tertiary transfections from the same secondary DNA generated tumors, the second group of tertiary tumors was named with a new letter and number designation, ie. I921-3 and O-14-1 were both tertiary tumors of I921 from different transfection events. Two representative Southern blots of the I-9 series tumor DNAs are shown in Fig.7. The BLUR8 probe hybridized to many fragments in I91, I92, and I93 primary tumor DNAs (data not shown). Three Alu positive restriction fragments were present in both secondary tumor DNAs tested (panel A, lanes I92-1, I92-3), but the human sequences were not transferred to the tertiary tumor, I921-3. Southern blot hybridization with a mouse H-ras cDNA probe (panel B) revealed a new fragment at approximately 11 kb in the secondary (I92-1 and I92-3), and tertiary (I921-3) DNAs. Since this fragment was not

TABLE 3. Tumorigenesis Analysis of Insulinoma I Series DNAs

TRANSFECTED TUMOR FORMATION			HYBRIDIZATION		WESTERN
DNA	Incidence	Onset ^a (weeks)	Alu	<u>ras</u>	Ras ^b
PRIMARY POOLS ^c					
I2	5 / 5	8	+	+	nd ^d
I6	5 / 5	11	+	+	-
I9	5 / 5	5	+	-	-
SECONDARY TUMORS					
I21	1 / 4	11	nd	nd	nd
I22	5 / 5	6	-	-	nd
I22 (repeat)	0 / 5	-			
I62	4 / 4	5	+	-	nd
I92	3 / 4	5 - 7	+	+	+
I93	0 / 5	-			
I95	0 / 5	-			
TERTIARY TUMORS					
I921	5 / 5	4	-	+	+
I921 (repeat)	5 / 5	4	-	+	+
CONTROLS					
T24 <u>ras</u>	5 / 5	3	+	+	+
pSV2neo	2 / 14	15	-	-	-

- a Time between injection of mice with transfected NIH 3T3 cells and detection of first palpable tumor. If all mice within a group did not develop tumors within the time of onset, then a range is given representing the time of onset from the first to last tumor.
- b Detection of p21 Ras by Western blot analysis.
- c Only pools inducing 2 or more primary tumors are shown; all primary tumorigenesis assays were done with 10 pools.
- d nd = Not determined.

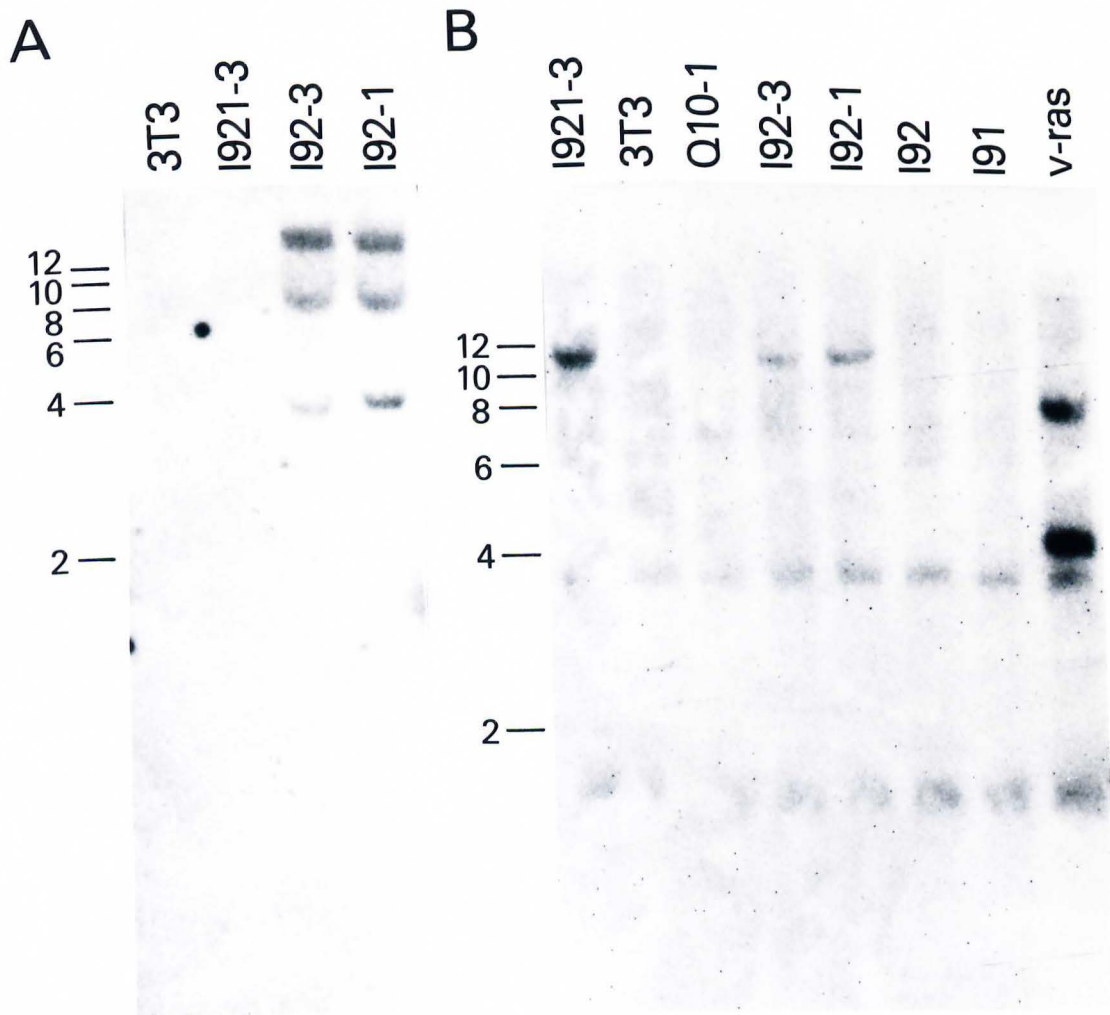


figure 7. **Southern blot analysis of I9-series nude mouse tumor DNAs.** **Panel A.** 10 μ g of each DNA was digested with EcoRI and the blot was probed with the BLUR8 insert for human repetitive sequences. **Panel B.** 10 μ g of each DNA was digested with BamHI and the blot was hybridized with mouse c-H-ras cDNA (I6s). DNA samples were from: untransfected NIH 3T3 cells, 3T3; pool I9 primary tumors, I91, I92; secondary tumors induced by I92 DNA, I92-1, I92-3; a secondary tumor negative for ras sequences, Q10-1; a tertiary tumor induced by I921 DNA, I921-3; and NIH 3T3 cells transformed by cloned Ha-MSV DNA, v-ras. The relative migration of selected fragments from the 1 kb ladder (BRL) are indicated in kilobasepairs. The fragments just below the 4kb and 2kb markers in panel B represent endogenous mouse c-H-ras, the fragment at 11kb in lanes I921, I923 and I921-3 is the newly integrated c-H-ras.

present in the primary tumor DNAs, it was concluded that a co-transfected mouse DNA fragment, containing the H-ras gene, had integrated into the secondary tumors and was transferred to tertiary tumors. This gene was not closely linked to the integrated human DNA since the Alu positive sequences were not co-transferred to the tertiary tumors.

Since the secondary and tertiary tumors contained ras sequences integrated in the DNA, ras expression was analysed by Northern and Western blotting. Total RNA from several tumors and untransfected cells was separated in denaturing agarose gels, transferred to nylon membranes and sequentially hybridized with mouse H-ras and chicken β -tubulin cDNA probes (Fig.8, panels A and B respectively). No significant differences were detected in H-ras specific mRNA levels between the ras positive tumors (I924, O-14-1, O-14-2), and untransfected NIH 3T3 cells and tumors from spontaneous transformants that did not contain integrated ras sequences (neo, neo4). Hybridization with the β -tubulin probe in panel B shows that equivalent amounts of RNA were transferred in each lane.

Western blot analysis was performed on tumor proteins from the I9- series tumors to determine whether p21 Ras was expressed in the tumors containing the newly integrated ras fragment in the DNA. In contrast to the equivalent H-ras specific mRNA levels found in all of the I9- series tumors, p21 Ras was only detected in the secondary and tertiary tumors by Western blot analysis (Table 3). Northern blot, and Southern blot hybridizations with probes for v-K-ras and N-ras showed no difference between the tumors and controls (data not shown), thus ruling out activation of these genes as the source of increased p21 Ras in the tumors. Western blot analysis of the I9- series and the amplified N-ras induced tumor proteins were performed in parallel since monoclonal antibody Y13-259 recognizes these two proteins with similar affinities (Furth et al 1982). As shown in Fig.9, p21 Ras was not detected in the I92 and I95 primary tumors (lanes 9,10), untransfected NIH 3T3 cells (lane 6), and other tumors not containing transfected ras sequences (lanes 7,12,13). Ras proteins were equally well detected in both the I921 tumor (lane 8) and in the tumors containing amplified copies of human N-ras (lanes 1 through 5). A tumor induced by T24 bladder

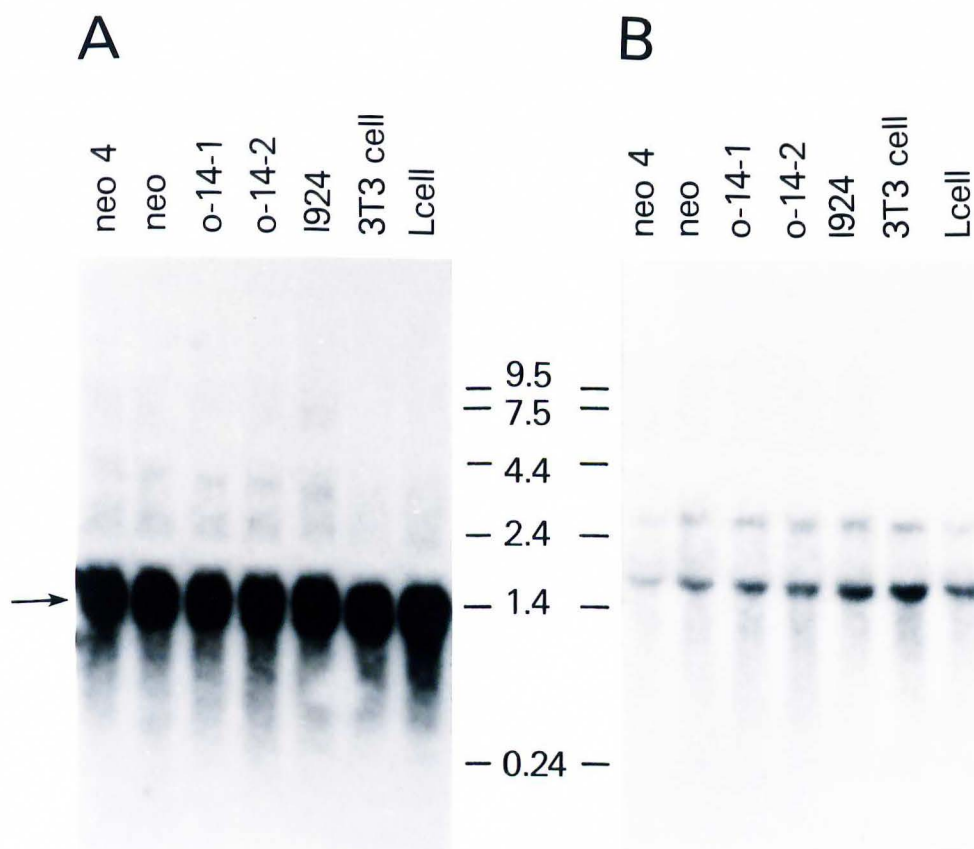


figure 8. **Northern blot analysis of tumor and cellular RNAs.** RNA samples, 15 μ g, were separated in a denaturing formaldehyde agarose gel, blotted and sequentially hybridized with mouse c-H-ras I6s cDNA probe in **Panel A**, and a chicken β tubulin probe in **Panel B**. RNA samples were extracted from untransfected L cells, L cell; NIH 3T3 cells, 3T3 cell; a secondary tumor, I924; two tertiary tumors induced by I921 DNA, O-14-1, O-14-2; and two spontaneous tumors generated from NIH 3T3 cells transfected with pSV2neo, neo, and neo4. Sizes of RNA molecular weight markers are indicated in kilobases.

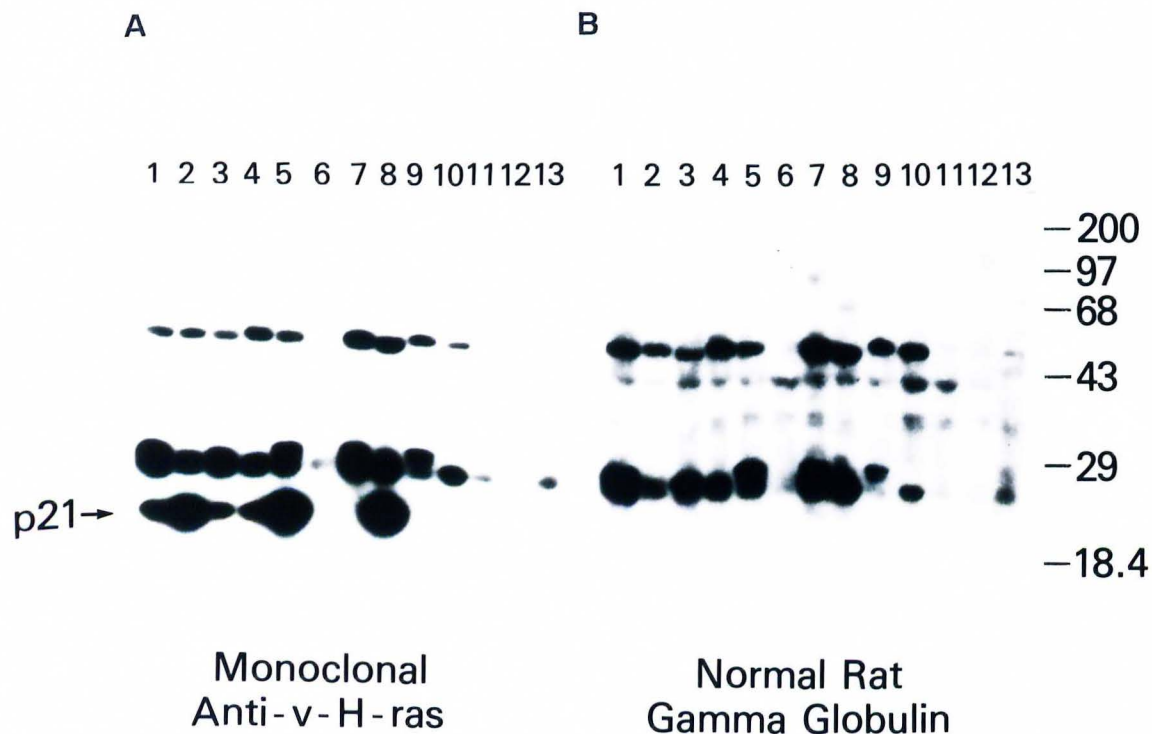


Figure 9. **Western blot analysis of tumor proteins for p21 Ras expression.** In **Panel A**, the first antibody was Y13-259, a rat monoclonal anti-ras antibody that cross-reacts with H-, N-, and K-ras proteins; **Panel B** first antibody was normal rat gamma globulin. Antibody reactivity was visualized with [125 I]protein-A coated with rabbit anti-rat antibodies. Protein samples include: **lanes 1 and 2**, tumors induced by λ cNras; **lanes 3, 4**, tumors induced by λ N21; **lane 5**, K series secondary tumor, K11-1; **lane 6**, NIH 3T3 cells; **lane 7**, primary tumor, I62; **lane 8**, secondary tumor induced from I92 DNA, I921; **lanes 9, 10**, pool I9 primary tumors, I95, I92; **lane 11**, positive control tumor induced by T24 DNA (codon 12 mutant human c-H-ras), **lanes 12, 13**, spontaneous transformant tumors that do not contain a transfected ras gene, Q10-1, and 3T3neo. The blots were over-exposed to show the p21 Ras in the positive control T24 induced tumor, lane 11. A shorter exposure of the left side of each blot is shown in Fig. 5. The size of prestained protein molecular weight markers is indicated at the right (X1000), and p21 Ras is indicated by an arrow.

carcinoma DNA which has a codon 12 mutant H-ras gene expressed barely detectable amounts of p21 (lane 11). Since the I9- series secondary and tertiary tumors expressed larger amounts of ras proteins, and arose with a longer latency than the codon 12 mutant H-ras induced tumors, a mechanism of H-ras activation other than mutation at the strongly transforming regions around codons 12 and 61 had to be considered. Two hypotheses were tested to explain the observation of increased p21 Ras without a concomitant increase in specific mRNA levels. The first hypothesis was that a stabilizing mutation in the expressed Ras protein caused an increased steady state concentration of Ras p21 which induced NIH 3T3 cells to become tumorigenic. The second hypothesis was that a non-coding mutation in the newly integrated gene caused the H-ras mRNA to be more efficiently translated.

To test these hypotheses, a cDNA library from poly(A) purified I921 secondary tumor RNA was made in lambda GT11 and H-ras cDNA clones were isolated and sequenced. The library contained 2×10^6 independent clones with 92% recombinants. Approximately 3×10^5 clones were screened with two H-ras specific probes: a HindIII fragment that includes most of the v-H-ras coding sequence (Ellis et al 1980), and a mouse c-H-ras 3' UTR specific oligonucleotide, CB65-1. Five H-ras positive cDNA clones were isolated and mapped with restriction enzymes. The three longest clones were subcloned into the EcoRI site of pGEM7zf + TM and sequenced directly using primers specific for plasmid sequences flanking the multiple cloning site and oligonucleotides specific for mouse H-ras sequences. A list of oligonucleotides used for sequencing can be found in Appendix F. The cDNA sequences were aligned and compared with a combination of sequences from the Genbank database using the sequence analysis software package of the Genetics Computer Group (Devereaux et al 1983) on a VAX 750 computer. Comparison of the cDNA sequences with sequence data from mouse v-H-ras BALB-MSV (Reddy et al 1985) and rat v-H-ras Ha-MSV (Dhar et al 1982), and a compilation of predicted viral and mammal H-ras amino acid sequences (Lacal et al 1988) indicated that there were no mutations in the cDNA coding sequences, thus ruling out mutational events that increased protein stability.

3. Alternative Splicing in Mouse c-H-ras mRNA

To test the second hypothesis, the 3' and 5' non-coding regions of the cDNAs were analysed for any differences that could account for a change in the translatability of the mRNA. The most striking differences between the sequences of the three cloned cDNAs were found in the 3'UTR. All three sequences diverged 6 bases 3' of the p21 termination codon (Fig. 10). The sequences surrounding the point of divergence were found to conform with the consensus rules for splice donor sites (Mount 1982). Inspection of the two sites where the sequences re-aligned, revealed that these areas resembled splice acceptor sites. Moreover, the areas of divergence and re-alignment (underlined in Fig. 10) coincided exactly with predicted splicing patterns. It was suspected that a previously undescribed intron was present in this region of the gene, and that alternative splicing of this intron gave rise to the various mRNAs from which the cDNA clones were derived. If this were true, then the various alignments of the cDNA clones with the mouse and rat viral H-ras sequences could be easily explained. First, mouse v-H-ras encoded by BALB-MSV was identical in this region to cDNA clone I10, containing the largest deletion (Fig. 10, compare MSV-ras with I10). This virus may have transduced the spliced form of the mRNA from the mouse. Second, the longest cDNA, I6s, was identical at 103 out of 110 nucleotides within the potentially spliced region, to the sequence of rat v-H-ras encoded by Ha-MSV (compare Rat-H-ras with I6s). As expected, this homology ends at the integration site between rat and viral sequences in Ha-MSV, so this virus seems to have transduced the unspliced form of the mRNA. The third cDNA clone, I76, was intermediate in length between I6s and I10 and may represent an alternatively spliced form of the H-ras mRNA that used a splice acceptor site 33 bases 5' to clone I10.

To determine whether alternative splicing occurs in the 3'UTR of mouse c-H-ras mRNA and whether the pattern of splicing is different between the tumor and normal cells, this area of H-ras mRNA was analysed by polymerase chain reaction amplification (PCR).

	781		840
Rat-H-ras	gTGTGTGCTG TCCTGACACC AGGTGAGGCA GGGACCAGCa AGACaTCTGG GGCAGTGgCC		
MSV-ras	ATGTGTGCTG TCCTGACACC AG.....		
I10	ATGTGTGCTG TCCTGACACC AG.....		
I76	ATGTGTGCTG TCCTGACACC AG.....		
I6s	ATGTGTGCTG TCCTGACACC <u>AGGTGAGGCA</u> GGGACCAGCG AGACGTCTGG GGCAGTGACC		
	end <u>CAG/GT</u>		
	841		900
Rat-H-ras	TCAGCTAGCC AGATGAACTT CATATCCACT tTGATGTCCc TGCTCCCCCA ATTCTGCCAA		
MSV-ras		
I10		
I76		
I6s	TCAGCTAGCC AGATGAACTT CATATCCACT CTGATGTCCCT TGCTCCCCCA ATTCTGCCAA		
	901		960
Rat-H-ras	TCCCCC.tGC CTGCA		
MSV-rasG CTCAGGACAT		
I10G CTCAGGACAT		
I76TCAG TCATGTCCTT TGTGCCCCGTC CCGGCACAGG CTCAGGACAT		
I6s	<u>TCCCCCCCCG</u> <u>CTGCAGTCAG</u> TCATGTCCTT TGTGCCCCGTC <u>CCGGCACAGG</u> CTCAGGACAT		
	<u>CAG/G</u>	<u>CAG/G</u>	
	961		1020
MSV-ras	GGAGGTGCCG GATGCAGGG		
I10	GGAGGTGCCG GATGCAGGGA GGAGGTGCCG ACGGAAGGAA GGAAAGAGGC GGAAGGAAG		
I76	GGAGGTGCCG GATGCAGGGA GGAGGTGCCG ACGGAAGGAA GGAAAGAGGC GGAAGGAAG		
I6s	GGAGGTGCCG GATGCAGGGA GGAGGTGCCG ACGGAAGGAA GGAAAGAGGC GGAAGGAAG		
	1021		1080
I10	GAAACGGTGC TGGAGCCAGG CCAGTCCAGG GATGGTGGAC AGATGTGACC AAGACCTTCG		
I76	GAAACGGTGC TGGAGCCAGG CCAGTCCAGG GATGGTGGAC AGATGTGACC AAGACCTTCG		
I6s	GAAACGGTGC TGGAGCCAGG CCAGTCCAGG GATGGTGGAC AGATGTGACC AAGACCTTCG		
	1081		1140
I10	CATGGACAAT TTGAACAGAC TGTCATGAAC TGTCCCTGTT GCCACTGGCA CCCAAGTCCT		
I76	CATGGACAAT TTGAACAGAC TGTCATGAAC TGTCCCTGTT GCCACTGGCA CCCAAGTCCT		
I6s	CATGGACAAT TTGAACAGAC TGTCATGAAC TGTCCCTGTT GCCACTGGCA CCCAAGTCCT		
	1141		1200
I10	CCACCCCTCT CAGTTCCTCC GGGCGCCTGC CTGTGAGGGC ACACGTTGCA TCACAGTAAA		
I76	CCACCCCTCT CAGTTCCTCC GGGCGCCTGC CTGTGAGGGC ACACGTTGCA TCACAGTAAA		
I6s	CCACCCCTCT CAGTTCCTCC GGGCGCCTGC CTGTGAGGGC ACACGTTGCA TCACAGTAAA		
	1201		1259
I10	TTATTTGATG GTCAAAAA..		
I76	TTATTTGATG GTCAAAAA36		
I6s	TTATTTGATG GTCttAAA39		

Figure 10. Comparison of the 3' untranslated regions of c-H-ras cDNAs. Sequences of clones isolated from the I92-1 cDNA library, I10, I76, I6s, are aligned with Genbank derived sequences: rat v-H-ras from HaMSV (Genbank:MHSp21) is labeled Rat-H-ras, and mouse v-H-ras from BALB-MSV (Genbank:MSVRAS) is labeled MSV-ras. Dots were added for alignment and the differences between mouse and rat are indicated by lower case letters in the rat sequence. Numbering is relative to the I6s clone as in figure 16. The stop codon signaling the end of p21 Ras translation is italicized, the putative splice donor and acceptor sites are underlined, and the consensus splice sequences are indicated.

Oligonucleotide primers were synthesized complementary to sequences 5' and 3' of the spliced area. A third oligonucleotide specific for sequences within all of the predicted amplified fragments was used as a probe to detect the specific amplified products. Cellular RNA from several tumors and cellular and cytoplasmic RNA from cell lines were reverse transcribed and the area of interest in the H-ras message was amplified by PCR. The amplified products were separated by electrophoresis through 5 % polyacrylamide gels, transferred to nylon membranes and hybridized with an H-ras specific oligonucleotide probe. The results of several independent amplifications are shown in Fig. 11. Samples in the right half of the figure were amplified from whole cell RNA except where indicated. Since the observed alternative splicing could be due to partially spliced forms of the RNA found in the nucleus and not in mature mRNA, cytoplasmic RNA from NIH 3T3 cells and L cells was also amplified (lanes: 3T3 cyt., L-cell cyt.). The same pattern of alternative splicing was detected in all RNA samples thus ruling out this possibility and supporting the suspected alternative splicing of this previously undescribed intron E. The upper 415 bp amplified fragment, represented by clone I6s, was the same size as the product amplified from genomic DNA and therefore corresponds to an unspliced form of the mRNA. As judged by the relative hybridization signals, this unspliced form represents a significant fraction of the H-ras mRNA in all samples, including cytoplasmic RNA from normal NIH 3T3 cells. The 268 bp amplified fragment, corresponding to the spliced form of the mRNA, was represented by clone I10 (lane p1019). This fragment appears to be derived from another significant part of the mRNA population in both tumor and normal cells. The 301 bp fragment amplified from clone I76, the one predicted to represent an alternatively spliced form of the H-ras mRNA, appears to represent a minor fraction of the H-ras transcripts. In some lanes this fragment is not visible in the figure, however it was represented in the amplification products of all RNAs tested. The lower abundance of this form of the mRNA may be due to inefficient recognition of a splice acceptor site that deviates from consensus.

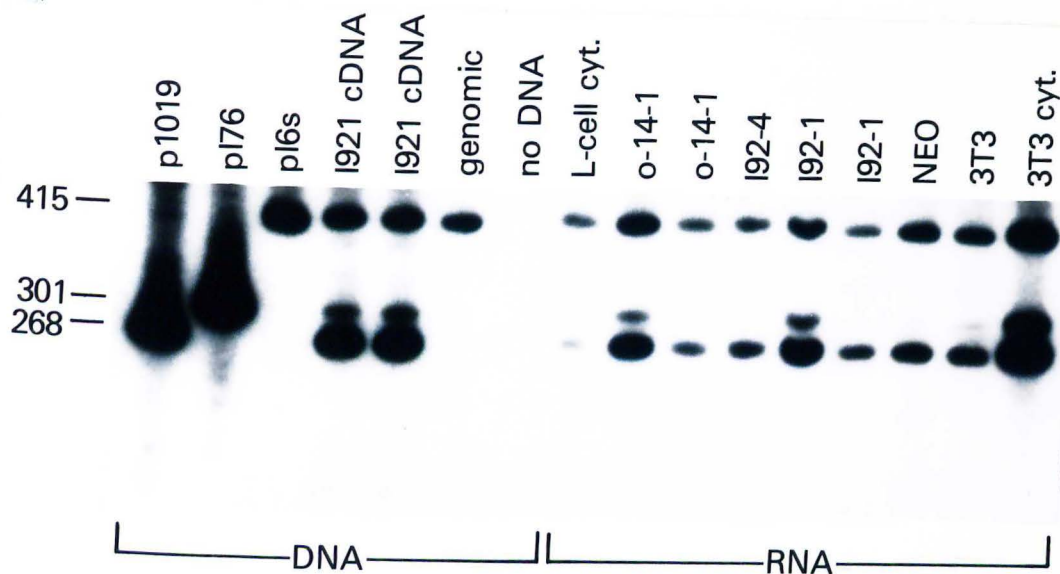


Figure 11. **Polymerase Chain Reaction (PCR) amplification analysis of alternative splicing at intron E of mouse c-H-ras.** Reverse transcribed RNA samples and DNA controls were amplified by 30 cycles of PCR. The amplified fragments were separated by electrophoresis in a 5 % polyacrylamide gel, blotted and hybridized with a [32 P]labeled oligonucleotide, CB63, to detect H-ras specific amplified fragments. Reverse transcribed RNA samples included cytoplasmic RNA from: NIH 3T3 cells and L cells, 3T3 cyt., L-cell cyt.; and whole cell RNA from: NIH 3T3 cells, 3T3; a tumor derived from spontaneous transformants transfected with pSV2neo, neo; two secondary tumors induced by I92 DNA, I92-1 (two separate amplifications), I92-4; a tertiary tumor induced by I921 DNA, O-14-1 (two separate amplifications). Control DNA samples included: plasmid subclones of the mouse H-ras cDNAs isolated from the I921 library, p1019 (elsewhere referred to as I10), p176, pl6s; Two aliquots of an unpackaged portion of the I921 cDNA library, I921 cDNA; genomic DNA from untransfected NIH 3T3 cells, genomic; and a sample amplified with no added template. The sizes of the amplified fragments are indicated in kilobasepairs.

The relative abundance of all three forms of the the H-ras mRNA were approximately equivalent in all tumor and normal cell RNAs tested.

Alternative splicing within intron D of the human H-ras gene was recently shown to have an effect on expression and transforming efficiency of a codon 12 mutant gene (Cohen et al 1989). The authors demonstrated that alternative splicing within intron D (between exons 3 and 4) results in inefficient processing of the mRNA. The putative truncated form of H-ras protein encoded by these transcripts is of unknown function and its existence was not proven. However, loss of the splicing mechanism by mutations within the intron resulted in increased expression of p21. To test whether alternative splicing also occurs within this intron in mouse c-H-ras and whether loss of this alternative splicing mechanism could have been the activating event in the nude mouse tumors, RNAs were amplified by PCR at intron D. Alternative splicing does occur at intron D in a minor fraction of mouse H-ras mRNAs as shown in Fig. 12. The 150 bp fragment representing the bulk of the mRNA is amplified from the spliced form; pl6s, and all of the cDNA clones isolated from the tumor library were in this conformation. The faint un-numbered band between 150 and 490 may be an artifact since it was amplified from both plasmid DNA and RNA. The fragment amplified from genomic DNA was 740 bp and was used to show the size of any unspliced transcripts or contaminating DNA. The 490 bp fragment amplified in all of the RNA samples may represent alternatively spliced mouse H-ras mRNA since it is 250 bp smaller than the fragment amplified from genomic DNA. The 490 bp fragment represents a low abundance form of the mRNA as had been reported for the human alternatively spliced transcript, but unlike the human and rat, this potential alternatively spliced intron is approximately 340 bases long. In both human and rat the reported size is smaller, 80 and 89 bases respectively (Cohen 1989). This 490 bp fragment is present in both untransfected NIH 3T3 cell and tumor RNAs, ruling out the loss of alternative splicing in this region as an explanation for increased H-ras expression in the tumors.

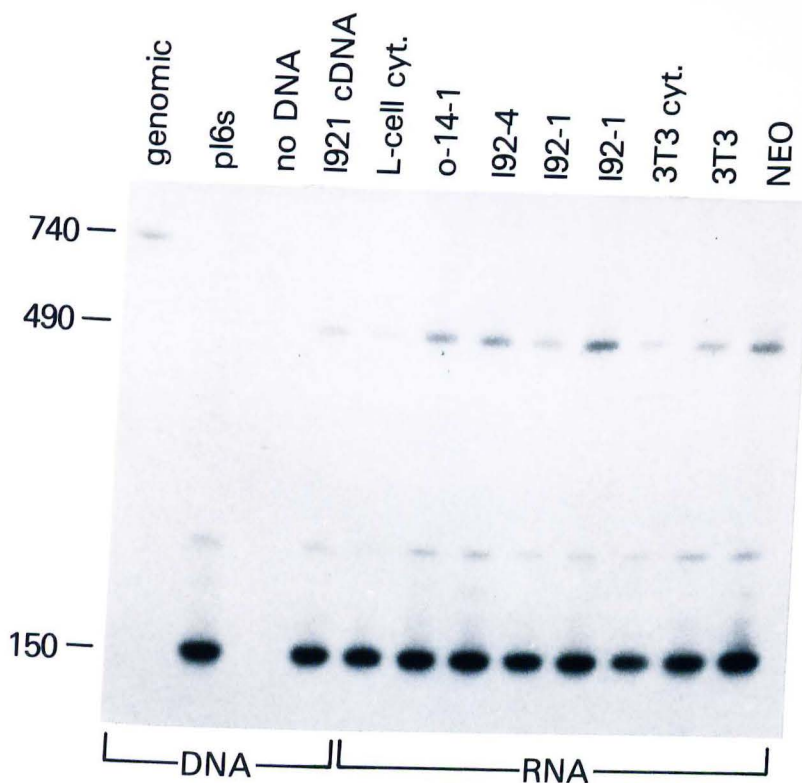


figure 12. **Polymerase Chain Reaction (PCR) amplification analysis of alternative splicing at intron D of mouse c-H-ras.** Reverse transcribed RNA samples and DNA controls were amplified by 30 cycles of PCR. The amplified fragments were separated by electrophoresis in a 5 % polyacrylamide gel, blotted and hybridized with [32 P]labeled oligonucleotide, H5-2, to detect H-ras specific amplified fragments. Reverse transcribed RNA samples included cytoplasmic RNA from: NIH 3T3 cells and L cells, 3T3 cyt., L-cell cyt.; and whole cell RNA from: NIH 3T3 cells, 3T3; a tumor derived from spontaneous transformants transfected with pSV2neo, neo; two secondary tumors induced by I92 DNA, I92-1 (two separate amplifications), I92-4; a tertiary tumor induced by I921 DNA, O-14-1. Control DNA samples included: a plasmid subclone of mouse H-ras cDNA isolated from the I921 library, pl6s; an aliquot of an unpackaged portion of the I921 cDNA library, I921 cDNA; genomic DNA from untransfected NIH 3T3 cells, genomic; and a sample amplified with no added template. The sizes of the amplified fragments are indicated in kilobasepairs.

In Vitro Translation

To test whether the presence of the alternatively spliced 3'UTRs have an effect on the efficiency of translation of the mRNA, the cDNA clones were linearized and transcribed in vitro using SP6 polymerase. The in vitro transcribed mRNAs were capped with guanylyl transferase and translated in rabbit reticulocyte extracts using [^{35}S]methionine. In pilot experiments 200 ng of mRNA, when translated for 1 hour at 30°C in 50 μl reactions, produced an easily detected signal. This was determined to be the concentration most likely to detect differences in translation efficiency, since twice as much RNA produced a proportionally larger signal. The identity of the translation products was verified by immunoprecipitation with the p21 specific monoclonal antibody Y13-259. As shown in Fig. 13, the translation products were immunoprecipitated specifically by the anti-ras monoclonal antibody, (lanes 2 through 7), and were not recognized by normal rat serum (lanes 8 through 11). No translation product was produced without the addition of exogenous mRNA (lane 1), and un-related translation products from the Brome Mosaic Virus were not immunoprecipitated (lane 8) in this system. The mRNAs were translated with, and without prior heat treatment, since some mRNAs require heat treatment to disrupt secondary structures that may inhibit translation initiation. Comparison of lanes 2,4,6 (heated) with lanes 1,3,5 (not heated) showed that heat pre-treatment did not have an effect on the translation rate of the H-ras mRNAs. A slight but reproducible difference in size was noted between the I6s and I76 translation products. Compared with molecular weight markers and co-electrophoresed Brome Mosaic Virus in vitro translation products, the apparent molecular weight of the I76 translation product was approximately 21,000 and the I6s product was approximately 23,000. Clone I10 mRNA produced a truncated in vitro translation product (lanes 6,7). As determined by sequence analysis, alternative splicing in intron C created a frame shift within the coding sequence in this clone which results in a truncated translation product from premature termination in exon 3. This truncated form of Ras is not efficiently immunoprecipitated by the monoclonal antibody since the epitope

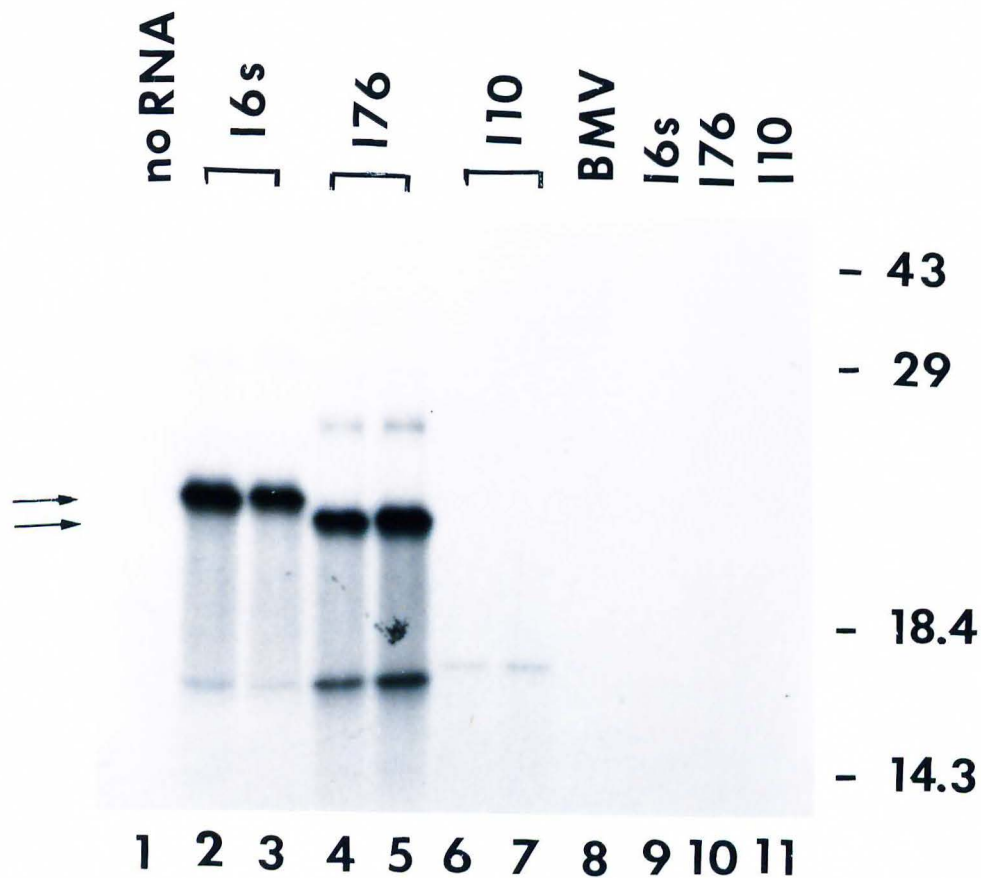


Figure 13. Immunoprecipitation of In vitro translation products with anti-Ras monoclonal antibody, Y13-259. In vitro transcribed and capped mRNAs (500 ng) from the mouse cDNA subclones I10, I76 and I6s, were translated in rabbit reticulocyte extracts with [35 S]methionine for 1 hour at 30°C in a 100 μ l reaction volume. An aliquot of each sample was immunoprecipitated by monoclonal antibody, Y13-259, (lanes 1-8) or normal rat gamma globulins (lanes 9-11) and protein-A sepharose coated with rabbit anti-rat antibodies. The immunoprecipitated proteins were separated by 12 % SDS PAGE under reducing conditions and visualized by fluorography. Duplicate RNA samples were either heated to 65°C prior to translation (lanes 2,4,6) or received no pre-treatment (lanes 3,5,7). The relative migration of prestained molecular weight markers is indicated at the right (X1000). The major translation products are indicated by arrows. The truncated protein produced by I10 is poorly recognized by the monoclonal antibody. Lanes 1 and 8 were translations run with no added RNA and with Bromo Mosaic Virus RNA respectively. Though the viral RNA produced several protein products, they were not recognized by the monoclonal antibody.

recognized by this antibody (aa 63-73) is close to the site of sequence divergence. When the translation products were run on SDS polyacrylamide gels without prior immunoprecipitation, the truncated protein was translated with the same efficiency as the other two clones (not shown). Since the predicted translation product of p110 would not bind GTP and could not be localized to the membrane, two prerequisites for ras protein function, this clone was not pursued further.

The apparent difference in size between the translation products of p16s and p176 could not be accounted for by any obvious changes in the coding sequences of the two cDNAs. To determine whether the molecular weight difference was due to undetected coding sequence changes, the clones were mapped by RNAase protection. Clone p16s was digested with BamHI and a full length antisense probe was synthesized using [$\alpha^{32}\text{P}$]CTP and T7 RNA polymerase. This probe was hybridized in solution to the *in vitro* transcribed mRNAs and the hybrids were digested with RNase to cleave the probe at sites of mismatches (Melton et al 1984). The products were separated on denaturing polyacrylamide gels and the protected fragments were visualized by autoradiography. No truncated fragments were detected (data not shown), as would occur if there were sequence mismatches between the clones. Since the protected fragments were not smaller than expected, coding sequence differences between the clones could not account for the differences in size of the translation products.

To determine whether the difference in size between the translation products was due to alternative splicing in the 3' UTR or to sequence differences in the 5' non-coding region, reciprocal constructs were made between the 5' UTRs, coding sequences and 3'UTRs of the cDNA clones. In vitro transcribed mRNAs from these chimeric clones were assayed by translation in rabbit reticulocyte extracts to determine which region of the mRNA was responsible for the different sizes of the translation product. The same amount of capped mRNA was translated and immunoprecipitated in all reticulocyte extracts. The structure of the chimeric cDNA clones and the respective translation products are summarized in Fig. 14.

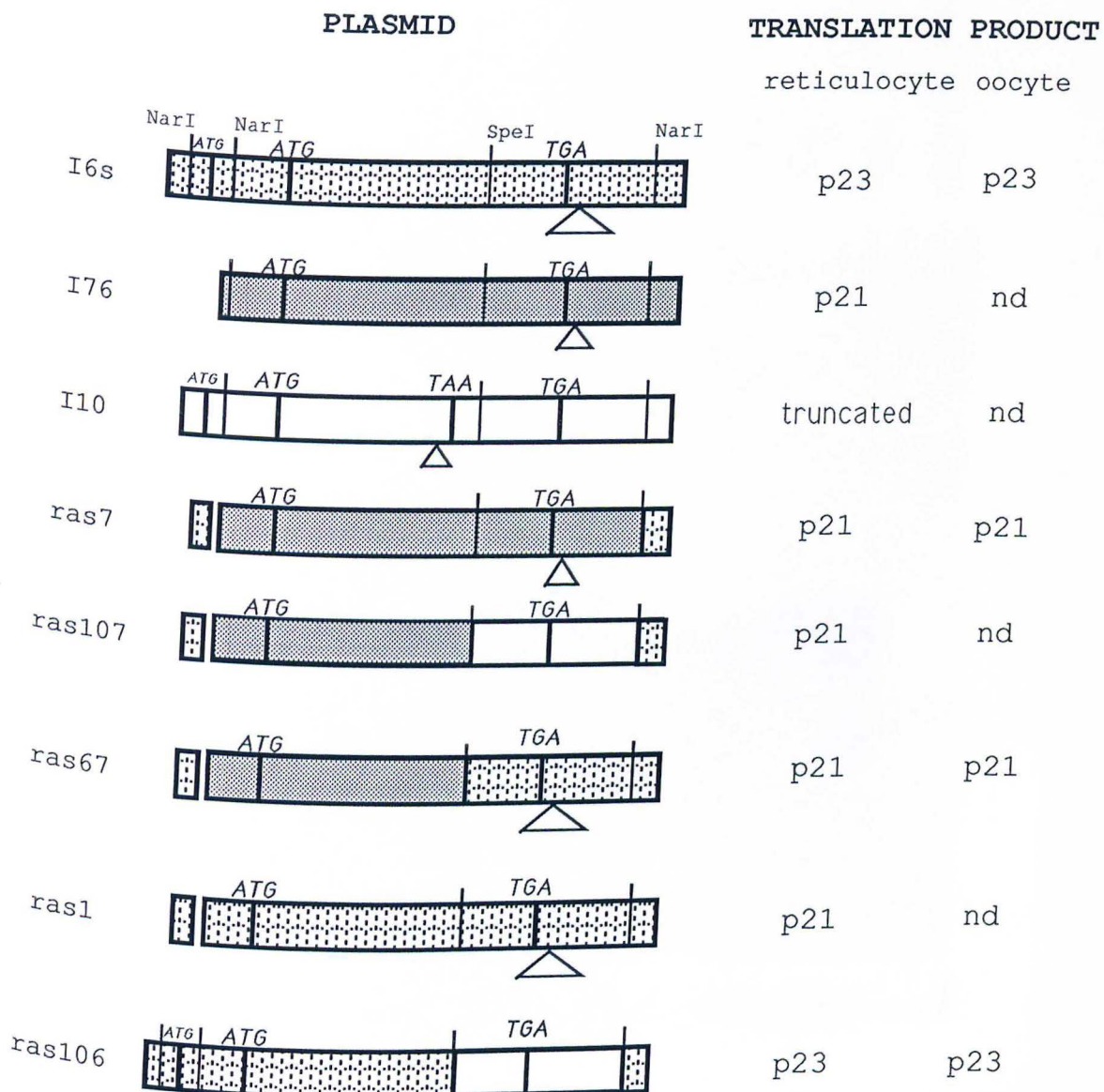


Figure 14. Schematic diagram of the structure of the c-H-ras chimeras and their translation products. The various sized triangles represent the alternatively spliced introns, and the transcription start sites and termination sites are indicated. The restriction enzyme sites used for construction of the chimeras are indicated above the first clone. The names of the chimeric plasmids are indicated to the left of each diagram. I6s, I76 and I10 are the original cDNAs clones, the others are derived from the indicated fragments of these clones. Capped SP6 transcribed mRNAs from the chimeric c-H-ras genes were translated in rabbit reticulocyte extracts or in *Xenopus* oocytes. The translation products are indicated under the system in which they were produced, nd = not determined.

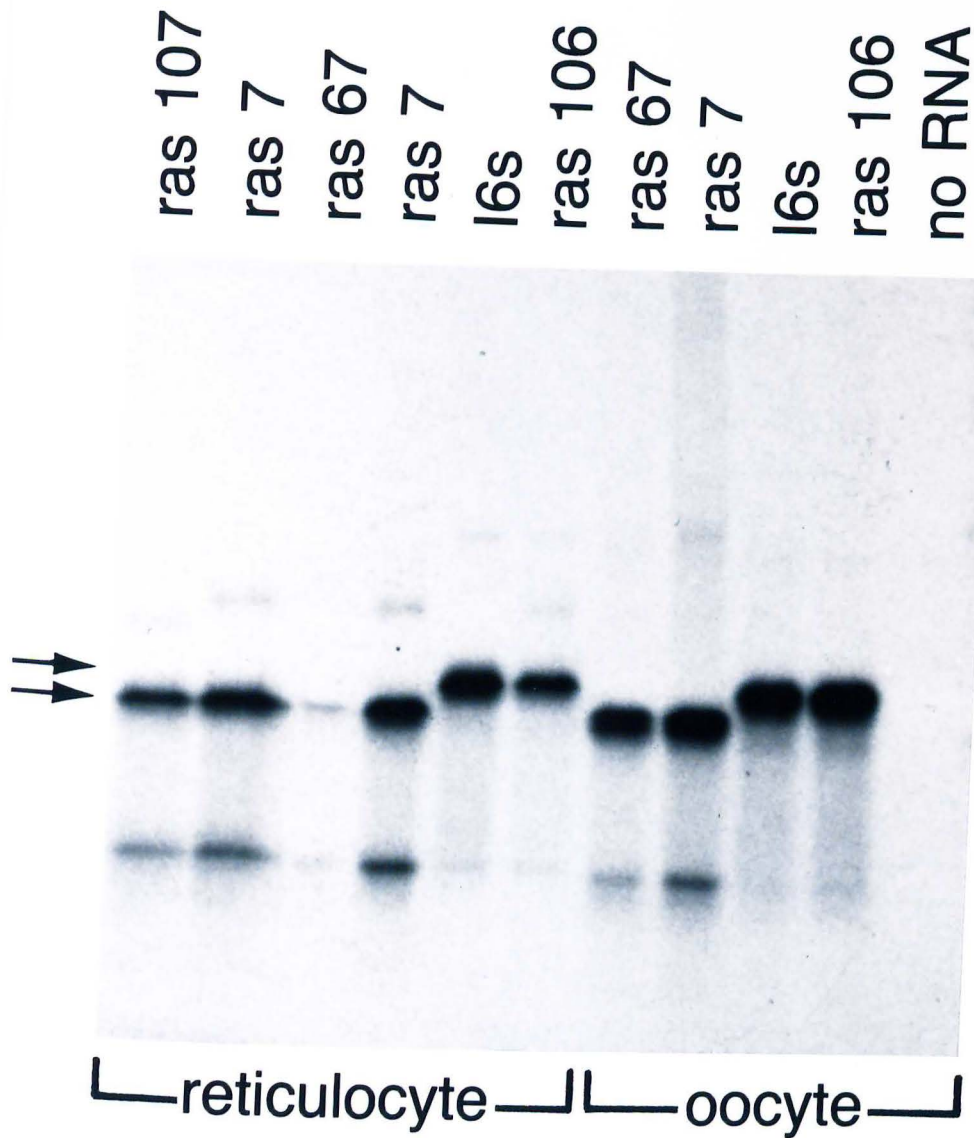


Figure 15. **In vivo and In vitro translation of chimeric c-H-ras mRNAs.** Translation products of In Vitro transcribed and capped chimeric mRNAs were immunoprecipitated with monoclonal antibody Y13-259 and the proteins were separated by 12 % SDS PAGE under reducing conditions. The chimeric plasmids used for mRNA production are indicated above each lane. RNAs translated in rabbit reticulocyte extracts are within the bracket labeled reticulocyte and the RNAs translated in Xenopus oocytes are within the bracket labeled oocyte. The different sizes of the translation products are indicated by arrows. The smaller band may be a degradation product.

The presence of the alternatively spliced 3'UTRs had no effect on the apparent size of the translation product, since ras7, ras107, and ras67 all had different 3' UTRs and all produced the same p21 product. The longer translation product was made only by those clones that contained the upstream 5' UTR sequences since the translation products of l6s and ras106 were p23 and the product of lras1, which has a 54bp deletion within the 5'UTR, was p21. To verify that the observed difference in size of translation products was not an artifact of the in vitro translation system, capped mRNAs from selected constructs were also translated in Xenopus oocytes. The same amount of each mRNA was injected into each oocyte, and the oocyte proteins were labeled by overnight incubation in [³⁵S]methionine. Protein extracts were made from pools of 20 oocytes injected with each mRNA and equivalent amounts of extracts, as determined by the BCA protein assay, were immunoprecipitated and separated by SDS polyacrylamide gel electrophoresis. Translation products of the same size as those of the reticulocyte extracts were seen when the mRNAs were translated in Xenopus oocytes (Fig. 15). Extracts from uninjected oocytes that were similarly treated did not contain immunoprecipitable [³⁵S]-labeled proteins (lane labeled no RNA). All chimeric mRNAs were translated with similar efficiencies within either system. The low signal in the reticulocyte system with ras67 in Fig. 15 was due to a calculation error, not to a real difference in translation efficiency.

Upstream ATG effects.

The 5'UTR sequences were compared between the cDNA clones, the normal mouse c-H-ras genomic sequence (Brown et al 1988), and the rat v-H-ras sequence from Ha-MSV (figure 16). The sequence between the two NarI sites at positions 9 and 65 contains an ATG translation initiation codon (italicized in the figure at position 50). Deletion of this NarI fragment invariably caused a shortening of the translation product. The original cDNA clone l76, which produced the shorter protein in reticulocyte lysates, does not contain the upstream ATG. Predicted translation of the normal H-ras sequence (Fig. 17), shows that the

```

1
rat H-ras      1 ..... .GCCCGCAGC CCGAGCCGCA CCCGCCGCGG ACGGAGCcCA 50
I10           ..... ..... ..... .CGCGG ACGGAGCTCA
I6s           CCGCTCCCCG CGCCCGCAGC CCGAGCCGCA CCCGCCGCGG ACGGAGCTCA
Genomic       CCGCTCCCCG CGCCCGCAGC CCGAGCCGCA CCCGCCGCGG ACGGAGCTCA
               NarI
50
rat H-ras      50 TGCGCGGGGCC CAGtCGGCGC CCGTCCGCGC CCCCGCCCTG CCCC GGCCCC 100
Msv-ras       ..... ..... ...TCCGCGC CCCCGCCCTG CCCC GGCCCC
I10           TGCGCGGGGCC CAGCCGGCG* CCGTCCGCGC CCCCGCCCTG CCCC GGCCCC
I76           GCGGGGCC CAGCCGGCG* CCGTCCGCGC CCCCGCCCTG CCCC GGCCCC
I6s           TGCGCGGGGCC CAGCCGGCG* CCGTCCGCGC CCCCGCCCTG CCCC GGCCCC
Genomic       TGCGCGGGGCC CAGCCGGCGC CCGTCCGCGC CCCCGCCCTG CCCC GGCCCC
               ORF> NarI
100
rat H-ras      100 GGCCCCG... ...GGGGCAG TCGCGCCAGC AAGCGGTGGG GCAaGAGCTC 150
Msv-ras       GGCCCCG.CC CCGGGGGCAG TCGCGCCAGC AAGCGGTGGG GCAGGAGCTC
I10           GGCCCCGGCC CCGGGGGCAG TCGCGCCAGC AAGCGGTGGG GCAGGAGCTC
I76           GGCCCCGGCC CCGGGGGCAG TCGCGCCAGC AAGCGGTGGG GCAGGAGCTC
I6s           GGCCCCGGCC CCGGGGGCAG TCGCGCCAGC AAGCGGTGGG GCAGGAGCTC
Genomic       GGCCCCGGCC CCGGGGGCAG TCGCGCCAGC AAGCGGTGGG GCAGGAGCTC
               ><intron A
150
rat H-ras      150 CTgGtTTGGC AGCCcCTGTA GAAGCgATGA CAGAATACAA GCTTGTGGTG 200
Msv-ras       CTAGATTGGC AGCCGCTGTA GAAGCTATGA CAGAATACAA GCTTGTGGTG
I10           CTGGATTGGC AGCCGCTGTA GAAGCTATGA CAGAATACAA GCTTGTGGTG
I76           CTGGATTGGC AGCCGCTGTA GAAGCTATGA CAGAATACAA GCTTGTGGTG
I6s           CTGGATTGGC AGCCGCTGTA GAAGCTATGA CAGAATACAA GCTTGTGGTG
Genomic       CTGGATTGGC AGCCGCTGTA GAAGCTATGA CAGAATACAA GCTTGTGGTG
               p21>

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Figure 16. **Comparison of c-H-ras 5' untranslated regions (UTRs).** The 5'UTRs of the cDNA clones I10, I76, and I6s were aligned with Genbank derived sequences: rat v-H-ras from Ha-MSV (Genbank:MHSp21) is labeled rat-H-ras, and mouse v-H-ras from BALB-MSV (Genbank:MSVRAS) is labeled MSV-ras; and with the mouse genomic c-H-ras sequence from Brown et al 1988, labeled Genomic. Dots were added for alignment and the differences between mouse and rat are indicated by lower case letters in the rat sequence. Numbering is relative to the I6s clone as in figure 10. The ATG start codons are in italics, the consensus sequences for initiation are underlined, as are the NarI restriction sites. Deletion of the NarI fragment in subclone ras1 results in translation of the smaller protein product. The initiation site of ras p21 is indicated by p21 > under the sequence. The site of splicing for intron 1 is indicated and the intronic sequences were removed from the genomic sequence for alignment. The single base deletion at position 69 is indicated with *.

1 CGCTCCCGGCGCCCGCAGCCCGAGCCGCACCCGCGCGGACGGAGCTCATGCGCGGGCCAGCCGGCGCCCGTCCGCGCC

upstream ORF>M R G P S R R P S A P

81 CCCGCCCTGCCCCGGCCCCCGCCCCGGGGGCGAGTCGCGCCAGCAAGCGGTGGGGCAGGAGCTCCTGGATTGGCA

P P C P G P G P G P G G S R A S K R W G R S S W I G S

161 GCCGCTGTAGAAGCTATGACAGAATAACAAGCTTGTGGTGGTGGGCGCTGGAGCGTGGGAAAGAGTGCCCTGACCATCCA

p21>M T E Y K L V V V G A G G V G K S A L T I Q

R C R S Y D R I O A C G G G R W R R G K E C P D H P

241 GCTGATCCAGAACCACCTTTGTGGACGAGTATGATCCACTATAGAGGACTCCTACCGGAAACAGGTGGTCATTGATGGGG

L I Q N H F V D E Y D P T I E D S Y R K Q V V I D G E

A D P E P L C G R V end

321 AGACATGTCTACTGGACATCTTAGACACAGCAGGTCAAGAAGAGTATAGTCCATGCGGGACCACTACATGCGCACAGG

T C L L D I L D T A G Q E E Y S A M R D Q Y M R T G

401 GAGGGCTTCTCTGTGTATTGCCATCAACAACACCAAGTCCTTCGAGGACATCCATCAGTACAGGGAGCAGATCAAGCG

E G F L C V F A I N N T K S F E D I H Q Y R E Q I K R

481 GGTGAAAGATTGAGATGATGTGCCAATGGTGTGGTGGGCAACAAGTGTGACCTGGCTGCTCGCACTGTTGAGTCTCGGC

V K D S D D V P M V L V G N K C D L A A R T V E S R Q

561 AGGCCCAGGACCTTGCTCGCAGCTATGGCATTCCCTACATTGAAACATCAGCCAAGACCCGGCAGGGCGTGGAGGATGCC

A Q D L A R S Y G I P Y I E T S A K T R Q G V E D A

641 TTCTATACACTAGTCCGTGAGATTGCGCAGCATAAATTGCGGAACTGAACCCACCCGATGAGAGTGGTCTGGCTGCAT

F Y T L V R E I R Q H K L R K L N P P D E S G P G C M

721 GAGCTGCAAAATGTGTGCTGCTCTGA

S C K C V L S end

Figure 17. Translation of normal c-H-ras. This sequence is a composite of sequences from the genomic sequence up to the second exon from Brown et al 1988, and the v-H-ras sequence from BALB-MSV, Genbank:MSVras. The translation initiation codons are underlined and the upstream out-of-frame open reading frame starts at -126 relative to p21 initiation, ends at +97, and is indicated by ORF >. The potential translation product of the ORF is underlined. The p21 Ras protein is indicated by p21 > and the sequence is truncated at the p21 translation termination codon.

1 CGCTCCCGGCGCCCGCAGCCCGAGCCGCACCCGCGCGGACGGAGCTCA^{*}TGCGCGGGCCAGCCGGCCGTCCTCCGCGCCC
fusion protein >M R G P S R R R P R P

81 CCGCCCTGCCCGGCCCCGGCCCCGGGGGCACTCGCGCCAGCAAGCGGTGGGGCAGGAGCTCCTGGATTGGCAG
R P A P A P A P A P G A V A P A S G G A G A P G L A A

161 CCGCTGTAGAAGCTATGACAGAATACAAGCTTGTGGTGGTGGGCGCTGGAGCGTGGGAAAGAGTGCCTGACCATCCAG
A V E A M T E Y K L V V V G A G G V G K S A L T I Q

241 CTGATCCAGAACCCTTTGTGGACGAGTATGATCCCACTATAGAGGACTCCTACCGAAACAGGTGGTCATTGATGGGGA
L I Q N H F V D E Y D P T I E D S Y R K Q V V I D G E

321 GACATGTCTACTGGACATCTTAGACACAGCAGGTCAAGAAGATATAGTCCATGCGGGACAGTACATGCGCACAGGGG
T C L L D I L D T A G Q E E Y S A M R D Q Y M R T G E

401 AGGGCTTCCTCTGTGTATTGTCATCAACAACACCAAGTCTTCGAGGACATCCATCAGTACAGGAGCAGATCAAGCGG
G F L C V F A I N N T K S F E D I H Q Y R E Q I K R

481 GTGAAAGATTGAGATGATGTGCCAATGGTGTGGTGGGCAACAAGTGTGACCTGGCTGCTCGCACTGTTGAGTCTCGGCA
V K D S D D V P M V L V G N K C D L A A R T V E S R Q

561 GGCCAGGACCTTGCTCGCAGCTATGGCATCCCTACATTGAAACATCAGCCAAGACCCGGCAGGGCGTGGAGGATGCCT
A Q D L A R S Y G I P Y I E T S A K T R Q G V E D A F

641 TCTATACACTAGTCCGTGAGATTGCGCAGCATAAATTGCGGAACTGAACCCACCCGATGAGAGTGGTCTGGCTGCATG
Y T L V R E I R Q H K L R K L N P P D E S G P G C M

721 AGCTGCAAAATGTGTGCTGTCTGACACCAGGTGAGGCAGGGACCAGCGAGACGTCTGGGGCAGTGACCTCAGCTAGCCAG
S C K C V L S end >unspliced intron E-----

801 ATGAACTTCATATCCACTCTGATGTCCTTGCTCCCCCAATTCTGCCAATCCCCCGCCTGCAGTCAGTCATGTCCTTTG

881 TGCCCGTCCCGGCACAGGCTCAGGACATGGAGGTGCCGGATGCAGGGAGGAGGTGCCGACGGAAGGAAGGAAAGAGGCGG
-----<

961 GAAGGAAGGAAACGGTGTCTGGAGCCAGGCCAGTCCAGGGATGGTGGACAGATGTGACCAAGACCTTCGCATGGACAATTT

1041 GAACAGACTGTCATGAACTGTCCCTGTTGCCACTGGCACCCAAGTCTCCACCCCTCTCAGTTCCTCCGGGCGCCTGCCT

1121 GTGAGGGGCACACGTTGCATCACAGTAAATTATTTGATGGTCTTAAAAA37

Figure 18. **Translation of clone I6s.** The complete sequence of cDNA clone I6s is presented, the unspliced intron E in the 3' untranslated region is indicated by >--<, the translation initiation codons are underlined. A single base deletion at position 69 (indicated by a *) changes the reading frame of the upstream ORF to make it coincide with translation of p21 Ras and a fusion protein is predicted. The fusion protein is double underlined up to the p21 translation initiation site (indicated by p21 >).

upstream ATG is out-of-frame with respect to the p21 coding sequence. The upstream ATG can potentially initiate translation of an open reading frame (ORF) that is 123 bases long and overlaps the p21 translation start site by 97 nucleotides. This ORF codes for a very hydrophilic putative protein product 74 amino acids long.

Translation of clone I6s starting from the upstream ATG predicts a fusion protein between the upstream ORF and H-ras p21 sequences (Fig. 18). The deletion of a cytosine at position 69 created a frame shift mutation and the reading frame of the upstream ORF became aligned with the ras coding sequence. The predicted length of this fusion protein is 231 amino acids, which is longer than expected if it is to account for the observed shift from p21 to p23 in the denaturing SDS polyacrylamide gels. Single point mutations have been shown to cause changes in the apparent molecular weight of Ras proteins when assayed under similar conditions (Srivastava et al 1985), so this discrepancy is not unprecedented. Kozak (1989) has recently shown that downstream secondary structure enhances the recognition of an upstream ATG. The sequence between the upstream ATG of c-H-ras and the first intron is approximately 96% G + C (Fig. 16) and has the potential to form several stem-loop structures. As shown in Figs. 13 and 15, the presence of the upstream ATG results in exclusive initiation at this site, with no initiation detected at the downstream ATG in either of the translation systems used. When the upstream ATG was removed from the mRNA as in constructs I76, ras7, ras107, ras67, and ras1, the normal ATG was used to efficiently initiate translation of p21. Since the upstream ATG is efficiently recognized in both translation systems to initiate translation of a ras fusion protein, this may also happen in vivo and constitute a novel mechanism for the overexpression of ras protein seen in the I921, and derivative tumors.

DISCUSSION

A. Tumorigenesis

Tumorigenesis is known to be a multi-step process (reviewed in Land et al 1983b). Similarly, transformation of normal cells in culture into tumor cells requires the cooperation of more than one activated oncogene (Newbold et al 1983, Ruley et al 1983, reviewed in Spandidos and Lang 1989). Oncogenes have been assigned to one of two complementation classes, the myc- or the ras class, based upon their ability to cooperate in the tumorigenic transformation of primary cell cultures (Land et al 1983a, Parada, et al 1984). One of the properties that has made the NIH 3T3 cell line the most used indicator for transforming genes is the fact that these cells are pre-neoplastic, and only require the action of a single activated oncogene to become tumorigenic (Cooper et al 1982). NIH 3T3 cell transfection assays for activated oncogenes have been designed to detect the presence of a single dominant oncogene in the donor DNA. Since NIH 3T3 cells transfected with activated ras-type oncogenes induce more rapidly growing tumors than those transfected with oncogenes from the myc class, the most dominant acting gene in a tumor DNA preparation masks the transforming activity of any other oncogene also present in the tumor. Fortuitously, in a few cases two activated oncogenes were detected in the same tumor (Murray et al 1983, Fasano et al 1984). The tumorigenesis assay was modified to increase the chance of detecting both strongly and weakly transforming oncogenes from the same original tumor. A preliminary sib-selection step was added to the bioassay for transforming genes described by Fasano et al (1984). This sib-selection procedure was also used by co-workers to detect an activated ras oncogene in human choriocarcinoma (Cohen et al 1988). The sib-selection step separates into pools individual colonies that have integrated different parts of the donor cell genome. The pools are then injected into different groups of mice for tumorigenesis analysis. Using this approach, pools containing potential weakly

transforming transfected genes can be separated from pools transfected with oncogenes which induce rapid tumor growth.

Renal cell carcinoma (RCC), and pancreatic beta cell insulinoma were the two tumor types tested for the presence of dominant acting oncogenes using the sib-selection tumorigenesis assay. Both of these malignancies have been associated with chromosomal abnormalities indicative of the loss of a tumor suppressor gene. The multiple endocrine neoplasia syndrome type I, a hereditary pre-disposition to develop endocrine tumors, including insulinoma, has been mapped to the loss of alleles on chromosome 1 (Mathews et al 1987). Both the sporadic and hereditary form of RCC are associated with chromosomal abnormalities on the short arm of chromosome 3 (Zbar et al 1987). Since tumorigenesis results from the loss of gene expression, these types of genetic abnormalities cannot be assayed by transfection of NIH 3T3 cells. The aim of these experiments was to determine whether any dominant acting oncogenes were also involved in the tumorigenic process in RCC and insulinoma. While these experiments were in progress, Fujita et al (1988) reported the detection of H-ras codon 12 mutation in a very low proportion of renal cell carcinomas (one out of fifteen). To date, no activated oncogenes have been associated with human insulinomas.

No novel dominant acting oncogenes were detected in the 5 tumor DNAs tested. The increased sensitivity of the tumorigenesis assay, as compared with the focus formation assay, allowed the detection of overexpressed ras proto-oncogenes activated during transfection. Overexpression of ras proteins resulting in tumorigenesis occurred by two profoundly different mechanisms.

B. Overexpression by N-ras Gene Amplification

Transfection and tumorigenesis analysis of a renal cell carcinoma DNA resulted in the production of primary nude mouse tumor DNAs containing approximately 50 copies of the human N-ras gene (Fig. 3). Secondary tumors induced by transfection of genomic DNA from a primary tumor also showed N-ras gene amplification, although to a lesser extent (approx. 20 copies). Analysis of full length phage clones isolated from a library of primary nude mouse tumor DNA showed that the clones efficiently induced tumors but not focus formation. A codon 61 mutant N-ras minigene used as a positive control in these experiments induced faster growing tumors and produced easily distinguishable foci. Mutations at codon 13 of N-ras has been reported to induce tumors but not produce foci when transfected into NIH 3T3 cells (Bos et al 1985, Hirai et al 1987). The possibility that this, or a similar mutation had activated the gene was ruled out by subcloning regions of the cloned N-ras gene and sequencing the coding exons. No point mutations were found in the coding sequence of the N-ras gene, though a few base changes and a small deletion were noted in the 3' untranslated region. DNA from a cloned normal human c-N-ras gene induced tumorigenesis with similar incidence and onset as compared with the gene cloned from the mouse tumor suggesting that the mutations detected in the 3' UTR probably had little to do with the observed tumorigenesis.

To determine whether the nude mouse tumors induced by transfection of human N-ras produced detectable ras protein, Western blot analysis of tumor proteins was done using a monoclonal antibody specific for p21 Ras. As shown in Fig. 5, p21 was overexpressed in N-ras induced tumors, including tumors induced by the lambda phage clone of normal human c-N-ras. In contrast, tumors induced by a codon 61 mutant N-ras minigene expressed 20 to 50 fold less p21 Ras, and no detectable levels of Ras protein were found in spontaneous NIH 3T3 cell tumors. Southern blot hybridization of genomic DNAs from the tumors induced by both control and test N-ras phage clones showed high copy numbers of integrated N-ras,

as had been detected in the primary and secondary nude mouse tumor DNAs. Southern blot hybridization of the original human tumor DNA and three metastases from the same patient showed that the N-ras gene was not amplified in the patient tissues (Fig.6). The most probable explanation of the data is that an amplification event had occurred during the primary transfection and tumorigenesis assay, and that integration and expression of amplified copies of transfected normal N-ras is sufficient to produce tumors in nude mice.

Several other groups have isolated N-ras from human tumors using nude mouse tumorigenesis assays. Fasano et al (1984) originally showed that amplified human N-ras could cause nude mouse tumors, although in those experiments the amplification was also present in the original tumor DNA. The amplified N-ras gene was cloned but not sequenced to rule out the possibility of concomitant amplification and mutation. In parallel transfections, however, the cloned normal c-N-ras was tumorigenic with the same incidence and onset as the amplified gene. Using a modification of the tumorigenesis assay described by Fasano et al (1984), another laboratory reported detection of human N-ras with a point mutation at codon 13 in acute myeloid leukemia DNA (Bos et al 1985). The mutant N-ras gene was amplified up to 30 fold in some of the primary nude mouse tumors, and in their experiments normal N-ras did not induce tumorigenesis. Hiarai et al (1987), using a modification of tumorigenesis technique used by Bos, reported the isolation of codon 13 mutant N-ras from myelodysplastic syndrome, an early stage of acute myeloid leukemia. Again, the authors reported that normal N-ras did not induce tumorigenesis.

In agreement with Fasano's results, the sib-selection tumorigenesis assay could detect amplified normal ras genes. The major difference between the two assays that showed no tumor formation from the normal N-ras gene and the two assays that showed normal N-ras induced tumorigenesis is the length of time the cells were kept in culture prior to injection into mice. The methods showing positive results used fully selected cells that were pooled and grown to mass culture for injection. The methods showing lack of tumor formation injected, pooled cells that had been in under selection for only 7 to 10 days after

transfection. The shorter pre-selection period may have resulted in injection of fewer cells expressing the co-transfected gene and a less sensitive assay. Also, comparison of the different assays showed a rough correlation between the amount of cells injected per mouse and the latency period for tumor formation. For example, injection of 1×10^7 cells resulted in development of positive control tumors within one week or less (Fasano et al 1984); when 2×10^5 cells were used, similar positive control tumors took 4 to 10 weeks to develop (Bos et al 1985). In the current study, injecting an intermediate amount of cells (2×10^6) resulted in positive control tumor formation in 2 to 3 weeks. Hiari et al (1987) used a similar inoculum (1.5×10^6) but did not report positive control results.

In an earlier study designed to correlate gene dosage and transformed phenotype, it was noted that when cells expressing low amounts of a mutant K-ras gene were injected into mice, the tumors that were produced contained amplified copies of the K-ras gene and produced more ras protein (Winter and Perucho 1986). The current work has also demonstrated that tumors arising from normal N-ras invariably show amplified copies of the transfected gene possibly more as a result of how much expression of the normal gene is required for tumorigenesis than an indication of gene amplification in the original tumor.

C. Alternative Splicing of c-H-ras

Southern blot analysis of the I9- series of nude mouse tumor DNAs revealed that a new copy of the mouse H-ras gene had co-integrated with human sequences during the secondary transfection event and, in two independent transfection experiments, was transferred to tertiary tumors. This gene was not highly amplified as had occurred with N-ras from RCC. Rather, overexpression of normal ras protein appears to have occurred by a novel mechanism in these nude mouse tumors. A new fragment hybridizing with a mouse H-ras cDNA probe was detected in secondary tumors at the same intensity as the normal H-ras fragments (Fig. 7). The newly integrated H-ras gene was tumorigenic since it induced

tertiary tumors in two separate experiments. This mouse H-ras gene was not closely linked to the integrated human DNA since the Alu positive sequences were not co-transferred to the tertiary tumors. To confirm that the newly integrated H-ras gene was expressed, the tumors were analysed by Northern and Western blotting. Surprisingly, no significant differences were detected in H-ras specific mRNA levels between the ras positive tumors, untransfected NIH 3T3 cells, and tumors from spontaneous transformants that did not contain integrated ras sequences. In contrast to the baseline levels of H-ras specific mRNA found in all of the I9- series tumors, p21 Ras was overexpressed in the secondary and tertiary tumors.

The I9- series secondary and tertiary tumors expressed approximately 20 to 50 times more ras proteins than tumors induced by codon 12 mutant H-ras. Ras protein overexpression combined with a latency period for tumor development of greater than two weeks was indicative of a mechanism of activation other than mutations at codons 12 or 61. As mentioned above, gene amplification was not involved in the overexpression of H-ras protein in the I series tumors. Also, the increased ras protein expression occurred without a concomitant increase in specific mRNA levels. Two hypotheses were tested to explain this discrepancy. The first hypothesis was that a stabilizing mutation in the coding sequence caused an increased steady state concentration of Ras protein which induced NIH 3T3 cells to become tumorigenic. The second hypothesis was that a non-coding mutation in the newly integrated gene caused the H-ras mRNA to be more efficiently translated. Comparison of the sequences from H-ras cDNAs isolated from an I921 secondary tumor cDNA library with published sequences from mouse and rat indicated that there were no mutations in the cDNA coding sequences, thus ruling out mutational events that increased protein stability.

To test the second hypothesis, the 3' and 5' non-coding regions of the cDNAs were analysed for any differences that could account for a change in translatability of the mRNA. The most striking differences between the the three cloned cDNAs were found in the 3'UTR. All three sequences diverged 6 bases 3' of the p21 termination codon (Fig. 10). The

sequences surrounding the point of divergence were found to conform with the consensus rules for splice donor sites (Mount 1982). Inspection of the two sites where the sequences re-aligned, revealed that these areas resembled splice acceptor sites. Moreover, the areas of divergence and re-alignment (underlined in Fig. 10) coincided exactly with predicted splicing patterns, suggesting that the cDNAs were derived from alternatively spliced mRNAs. Alternative splicing in the 3' UTR was confirmed by PCR amplification of RNAs from nude mouse tumors and untransfected NIH 3T3 cells. As shown in Fig. 11, a similar alternative splicing pattern was seen in all RNA samples tested. This is the first time that alternative splicing has been directly demonstrated at this intron, providing a possible explanation for the differences between the 3' untranslated regions of published sequences of H-ras cDNAs. The sequences of mouse and rat v-H-ras diverge sharply within 6 bases of the stop codons. This was previously attributed to genetic drift within non-coding regions of the gene; it now seems obvious that the apparent divergence resulted from alternative splicing. Within the 3'UTR, rat H-ras mRNA transduced by Ha-MSV is approximately 90 % identical to the unspliced form of the mouse H-ras mRNA, represented by clone I6s. As expected, the sequence similarities end at the junction between rat and viral sequences in Ha-MSV. Therefore, it was the unspliced form of the rat H-ras mRNA that was transduced by Ha-MSV. The spliced form of the mouse H-ras mRNA was transduced by BALB-MSV, since the viral sequence is identical to clone I10 in this region (Fig. 10). The alternatively spliced form of the H-ras mRNA has not been transduced by a virus and is reported for the first time in this investigation. The possibility of alternative splicing in the 3'UTR of human H-ras was alluded to recently (Cohen et al 1989). The authors noted that their H-ras cDNA clones were missing 106 nucleotides within the 3' UTR in the same region detected in this study. This may be evidence for conservation of the 3' UTR alternative splicing mechanism through evolution.

The relative abundance of all three alternatively spliced forms of the H-ras mRNA was the same in all RNA samples tested. The unspliced and spliced forms were approximately

equivalent and together represented the vast majority of the H-ras mRNA in all samples. The alternatively spliced form of the H-ras mRNA appeared to represent a minor fraction of the H-ras transcripts. The lower abundance of this mRNA form may be due to inefficient recognition of a splice acceptor site that deviates from consensus. This acceptor site has a T instead of a G at the base following the critical AG in the splice consensus sequence (Aebi et al 1986). Although this is a less conserved base, only 8% of functional splice acceptor sites have a T at this position (Mount 1982). As discussed below, defective processing may be a mechanism of reducing the concentration of mature H-ras mRNAs available for translation.

Alternative splicing that suppresses gene expression has recently been shown to occur in the human H-ras mRNA. The alternative exon, IDX, within intron D of the Human c-H-ras gene acts as a down regulator of H-ras expression by a possible mechanism of defective splicing (Cohen et al 1989). Deviation from consensus at the 5' splice junction can interfere with the second step of splicing and result in defective intermediates that are lost during processing (reviewed in Aebi and Weissmann 1987). The 5' splice junction of the IDX is not consensus and splice reactions involving this donor are not accumulated in the cell. Point mutations in either splice junction of the IDX abolishes alternative splicing and up-regulate H-ras expression approximately 10 fold (Cohen et al 1989). To test whether alternative splicing occurs at the mouse c-H-ras intron D in normal cells and whether H-ras overexpression in the mouse tumors was due to lack of alternative splicing, RNA from normal NIH 3T3 cells and several tumors was reverse transcribed and the splice junction at intron D was amplified by PCR. As shown in Fig. 12, alternative splicing does occur at intron D in a minor fraction of mouse H-ras mRNAs. The low abundance of the alternatively spliced transcript would be predicted by the mechanism of defective splicing proposed by Cohen et al (1989). The mouse alternatively spliced intron D is approximately 340 bases long as estimated by the relative sizes of the spliced and alternatively spliced amplified fragments. In both human and rat the reported size is smaller, 80 and 89 bases respectively (Cohen

1989). The differences in size suggest that the mechanism of defective splicing is more important than the sequence of the encoded alternative translation product. Since the relative abundance of the 490 bp amplified fragment was similar in RNA samples from both untransfected NIH 3T3 cells and ras induced tumors, loss of alternative splicing in this region cannot be the explanation for increased H-ras expression in the tumors.

To test whether the presence of the alternatively spliced 3'UTRs have a direct effect on the efficiency of translation of the mRNA, chimeric constructs containing the different 3'UTRs were transcribed in vitro, and the mRNAs were capped and translated in rabbit reticulocytes. The identity of the translation products was verified by immunoprecipitation with the p21 specific monoclonal antibody Y13-259. No correlation between 3'UTRs and translation efficiency could be made. As expected, clone I10 produced a truncated protein. Sequence analysis revealed that this cDNA clone was potentially alternatively spliced at intron C (again with a T next to the conserved AG of the 3' acceptor site as detected in the alternatively spliced form of the 3'UTR), and translation through the alternatively spliced region produced a frame-shift in the coding sequence and premature termination in exon 3. This truncated form of Ras was not efficiently immunoprecipitated by the monoclonal antibody since the epitope recognized by this antibody (aa 63-73) is close to the site of sequence divergence. The mRNA coding for the truncated product was translated with the same efficiency as the other mRNAs tested. Since the predicted translation product of pI10 should neither bind GTP nor be localized to the membrane, two prerequisites for ras protein function, this clone was not studied further.

A slight but reproducible difference in size was noted between the I6s and I76 translation products that could not be explained by sequence analysis. After confirming that the clones were identical in the coding sequence by RNAase protection mapping, several reciprocal constructs were made between the different regions of the cDNAs to establish a correlation between the size of the translation product and the region of the cDNA included in the construct. Constructs containing the different alternatively spliced 3' UTRs produced

the same translation product when the 5' UTR was kept constant. As shown in Fig. 14, the larger translation product, p23, was only made by clones containing 5' UTR sequences within a 54 bp *NarI* fragment. To rule out the possibility that the observed difference in size could be an artifact of the reticulocyte translation system, selected chimeric mRNAs were also translated by injection of Xenopus oocytes. As shown in Fig. 15, the translation products were identical in vivo and in vitro, and all chimeric mRNAs were translated with similar efficiencies within either system.

C. Translation Initiating at an Upstream ATG

An upstream ATG was identified within the *NarI* fragment required for translation of the p23 product. Since the cDNA clone I76 was not full length, the upstream ATG was missing and the shorter translation product was produced in reticulocyte lysates. The predicted translation product of clone I6s initiated at the upstream ATG is a fusion protein between the upstream open reading frame (ORF) and H-ras p21 sequences (Fig. 18). In contrast, the upstream ORF from the published sequence of normal mouse c-H-ras is out of frame with respect to p21. The normal c-H-ras upstream ATG potentially initiates translation of an ORF that is 123 bases long and overlaps the p21 translation start site by 97 nucleotides. This ORF codes for a very hydrophilic putative protein product 74 amino acids long. The deletion of a cytosine at position 69 in clone I6s, created a frame shift mutation and the reading frame of the upstream ORF became aligned with the ras coding sequence. The predicted length of this fusion protein is 231 amino acids, which is longer than expected if it is to account for the observed mobility shift from p21 to p23 in the denaturing SDS polyacrylamide gels. Single point mutations have been shown to cause changes in the apparent molecular weight of Ras proteins when assayed under similar conditions (Srivastava et al 1985). Therefore, discrepancies in the apparent molecular weight of H-ras proteins are not unprecedented.

In eucaryotic organisms, the ATG closest to the cap site is preferentially used for initiation of translation (reviewed in Kozak 1989). In a survey of 699 vertebrate mRNAs, 90 % followed the "first ATG" rule (Kozak 1987a). Proto-oncogenes are the one group noted for the presence of upstream ATGs. It has been postulated that these upstream ATGs may be involved in modulation of proto-oncogene expression (Kozak 1987a, Marth et al 1988). The survey also revealed that the context of the ATG is important for recognition as a translation initiation site and a consensus sequence was proposed (Kozak 1987a). Mutational analysis of the consensus sequence has demonstrated that purines at -3, -6, and -9 and the G at +4 are important for efficient recognition (Kozak 1987b). Recently, Kozak (1989) has shown that downstream secondary structure enhances the recognition of an upstream ATG that is otherwise not in good context for recognition. According to the consensus sequence, the upstream ATG in the chimeric H-ras cDNAs is in a relatively poor context for recognition, with the critical -3 purine absent; however, the sequence between the upstream ATG of c-H-ras and the first intron is approximately 96% G + C (Fig. 16) and has the potential to form several stem-loop structures. The very high G + C content of this area may cause preferential initiation from the upstream ATG. As shown in Figs. 13 and 15, the presence of the upstream ATG results in exclusive initiation at this site, with no initiation detected at the downstream ATG in either of the translation systems used. When the upstream ATG was removed from the mRNA, the normal ATG was used to efficiently initiate translation of p21. From the results of this study it may be predicted that if the upstream ATG is recognized, initiation of translation of a relatively long ORF which overlaps the p21 start site could decrease translation of p21 and be a mechanism by which the cell modulates H-ras protein expression. Reduced expression from downstream overlapped reading frames has been shown in both model systems (Peabody and Berg 1986, Peabody et al 1986), and in viruses (reviewed in Kozak 1989).

Most ras genes contain upstream ATG(s) (Kozak 1987a). Human N-ras contains 2 upstream ATGs and the upstream ORF potentially initiating at the second ATG terminates

within 18 bases of the p21 start site (Hall and Brown 1985). This second upstream ORF is conserved between human and guinea pig (Doniger 1987). The H-ras gene from several species contain at least one upstream ATG (**human**, Ishii et al 1985; **rat**, Damante et al 1987; **mouse**, Brown et al 1988). The K-ras 5' non-coding region in the mouse, as mapped by Hoffman et al (1987) does not contain an upstream ATG. Ribosomal recognition of the upstream ATG(s) in ras mRNA may be a general mechanism for modulation of ras expression in vivo. This hypothesis also provides an explanation for the observed discrepancies between the unchanged mRNA level and the overexpression of Ras protein in the nude mouse tumors. A point mutation in the 5' UTR causes the production of a fusion protein and circumvents the modulation of translation by the upstream ATG. The net result is an increase in production of a ras fusion protein without a concomittant increase in mRNA.

There are several examples of the production of functional amino terminal ras fusion proteins in the literature. H-ras has been expressed in E. coli as fusion proteins with an amino terminal extensions (Lautenberger et al 1983, Poe et al 1985). Bacterially expressed fusion proteins were fully functional in microinjection studies (Feramisco et al 1984, Stacey and Kung 1984). The viral Ra-SV encoded p29 is a gag-ras fusion protein (Gonda et al 1982); since v-Ra-ras was created by in-frame fusion of rat H-ras mRNA with a retroviral gag gene, this fusion protein and the predicted fusion protein from the nude mouse tumors are identical in 18 of 21 amino acids within the amino terminal extention. The p29 v-Ra-ras fusion protein is functional as an oncogene. Therefore the presence of an amino terminal extension should not interfere with the function of the ras protein translated from clone I6s. This remains to be proven, however, by transfection of NIH 3T3 cells with the I6s cDNA cloned into a suitable eucaryotic expression vector.

A recent report described the novel activation of murine H-ras by to a complex proviral insertion mechanism that resulted in increased expression of normal H-ras protein (Ihle et al 1989). MoMuLV integration in the intron between exon -1 and the first coding exon resulted

in tumorigenesis from overexpression of normal H-ras p21. This was attributed solely to increased transcription from the viral LTR. According to the hypothesis put forth in this thesis, the H-ras mRNA expressed from the LTR should be more efficiently translated due to the removal of exon -1 and the upstream ATG. Ihle et al did not address this possibility because, when looking for open reading frames in the -1 exon sequenced from their genomic clone, they translated through the first intron. Therefore, several termination codons were found within the intron and it was deduced that no upstream ORF existed in mouse c-H-ras. Translation of exons -1 and 1 from the genomic sequence reported by Brown et al (1988), as depicted in Fig. 17, shows a single long open reading frame upstream of, and overlapping the p21 start site.

A related mechanism of upstream ATGs modulating proto-oncogene expression has been proposed for translational control of the lck gene (Marth et al 1988). They showed that integration of MoMuLV within the intron between exon -1 and 1 of the lck gene caused increased mRNA transcription from the retroviral promoter, and increased translation efficiency by removing upstream ATG codons from the mRNA of the LSTRA lymphoma cell line. The increased translation efficiency shown by several constructs in which the upstream ATGs were either deleted or mutated provided support for their explanation. The presence of upstream ATGs in proto-oncogenes does not necessarily mean that they function as modulators of translation efficiency. The upstream ORFs in the long leader sequence of c-sis/PDGF were shown to have no effect on the translation of sis/PDGF protein (Rao et al 1988). Instead, the presence of a long G + C rich sequence within the 5' UTR was shown to be associated with inhibition of translation of this proto-oncogene.

Overexpression of Ras protein in the nude mouse tumors was accomplished by two mechanisms: 1- N-ras gene amplification, and 2- a single base deletion in the 5' UTR that is proposed to cause an upstream out-of-frame AUG which may normally serve to modulate gene expression, to become the initiation codon for an H-ras fusion protein that may therefore be better translated. Both of these activational events occurred during the

transfection and tumorigenesis assays. Amplification of transfected genes is a common occurrence. Injection of transfected cells into animals for tumorigenesis testing exerts selective pressure for the cell with the most transformed phenotype to make the tumor. Injection of cells containing low copy numbers of K-ras into syngeneic rats, resulted in the formation of tumors containing amplified K-ras (Winter and Perucho 1986). Overexpression of normal Ras proteins can cause NIH 3T3 cells to become tumorigenic by raising the number of GTP bound activated Ras molecules above the threshold necessary for uncontrolled signal transduction to occur (reviewed in Sistonen and Alitalo 1986, Spandidos and Lang 1989). Western blot analysis of the I9- series and the amplified N-ras induced tumor proteins were performed in parallel since monoclonal antibody Y13-259 recognizes these two proteins with similar affinities (Furth et al 1982). Similar amounts of ras protein were detected in both sets of tumors, giving an indication of the amount of normal ras protein required for tumorigenicity (Fig. 9). Approximately 20 to 50 fold less mutant ras protein expression is required for tumorigenesis. If overexpression of the ras proto-oncogene can cause tumorigenesis, and ras p21 has a 20 hour half-life, the cell must have several mechanisms to insure that this does not happen. Two mechanisms that may function to down regulate p21 expression are defective processing of alternatively spliced mRNAs as demonstrated by Cohen et al (1989) and addressed in this work, and modulation of translation through an upstream out-of-frame ATG. A novel mechanism of H-ras overexpression is proposed by which the second of these processes is circumvented.

Appendix A: Buffers for DNA analysis

STET (plasmid DNA)

8 % sucrose
5 % Triton X100
50 mM Tris, pH 8.0
50 mM EDTA
filter sterilized and stored at 5°C

RNAase stock (100 X)

600 µg/ml RNase A
600 U/ml RNase T1
boil 5 min., cool slowly, store 5°C

TNE (genomic DNA)

20 mM Tris, pH 7.5
20 mM NaCl
10 mM EDTA

TE buffer for DNA storage

10 mM Tris, pH 8.0
0.5 mM EDTA

5X STOP

0.1 M EDTA
0.1 % Bromphenol blue
10 % Ficoll

Denaturing solution

0.5 M NaOH
0.6 M NaCl

Denhardt's solution (100 X)

2 % Ficoll
2 % Polyvinylpyrrolidone
2 % BSA (optional)

SET (20 X)

3.0 M NaCl
0.6 M Tris pH 7.8
40 mM EDTA

Hybridization Solution III (oligos)

5 X SET
5 X Denhardt's, without BSA
0.5 % SDS
50 µg/ml sheared, denatured salmon sperm DNA (Loftstrand Labs)

TAE (50X)

(agarose gel electrophoresis buffer)
242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5M EDTA (pH 8.0)
1X running buffer is
40mM Tris-acetate, 1mM EDTA

Ligase buffer (10 X)

0.5 M Tris, pH 7.8
100 mM MgCl₂
100 mM Dithiothreitol
5 mM ATP
store at -20°C in aliquots

Universal restriction enzyme buffer (10 X)

330 mM Tris acetate, pH 7.9
660 mM Potassium acetate
100 mM Magnesium acetate
5 mM DTT
1 mg/ml BSA
store at -20°C in aliquots

Neutralization Solution

0.5 M Tris, pH 7.5
0.6 M NaCl

Hybridization Solution I

6 X SET
3 X Denhardt's solution
0.1 % SDS
30 mM sodium phosphate, pH 7.2
100 µg/ml sheared, denatured salmon sperm DNA (Lofstrand Labs, Gaithersburg, MD)

Hybridization Solution II

0.5 M Sodium phosphate, pH 7.2
0.5 g/ml Crystalline BSA
7 % SDS
1 mM EDTA
50 µg/ml sheared, denatured salmon sperm DNA (Loftstrand Labs)

Appendix B: Media and buffers for Library analysis

SM (phage storage buffer)

2.32 g NaCl
20 ml 1 M Tris, pH 7.5
3.2 ml 1 M MgSO₄
0.04 g Gelatin
deionized H₂O to 400 ml
filter and autoclave

NZCYM broth (1 liter)

10 g NZ Amine
5 g NaCl
5 g Yeast extract
1 g Casamino acids
dist. H₂O to 1 liter, pH 7.5 with NaOH,
autoclave, add 10 ml 1 M MgSO₄ last

Ampicillin stock (AMP)

25 mg/ml ampicillin (sodium salt)
in H₂O, 0.45 μ filter, store frozen

Luria-Bertani (LB) broth

10 g Bacto-tryptone
5 g Bacto-yeast extract
10 g NaCl
dist. H₂O to 1 liter, pH 7.5 with NaOH,
autoclave

Detection of β galactosidase

LB top agarose

0.5 mM IPTG (0.1 M stock in H₂O)
100 μ g/ml Xgal (20 mg/ml stock in N,N'dimethyl formamide)

TM (phage dilution buffer)

2.32 g NaCl
20 ml 1 M Tris, pH 7.5
3.2 ml 1 M MgSO₄
deionized H₂O to 400 ml,
filter and autoclave

LB bottom agar

500 ml LB broth
7.5 g Agar
autoclave, cool to 60°C add 100 μ g/ml AMP
if necessary and pour into plates

LB top agarose

100 ml LB broth
0.6 g agarose
autoclave, cool to 50°C add 1 ml 1M MgSO₄,
use 3 ml for 100mm plates,
use 6 ml for 150mm plates

Appendix C. Buffers for RNA Analysis

Solution D

4 M Guanidinium thiocyanate
25 mM Hepes, pH 7.0
0.5 % Sarcosyl
0.1 M β -Mercaptoethanol
(added before use)

Sucrose lysis buffer

140 mM NaCl
1.5 mM $MgCl_2$
10 mM Tris, pH 8.5
1.0 % NP40
24 % (w/v) Sucrose

High salt buffer

10 mM Hepes, pH 7.5
0.5 M NaCl
0.05 % SDS
1.0 mM EDTA

Formaldehyde agarose gels

1 % (w/v) Agarose
1 X MOPS buffer
2.2 M Formaldehyde
1 μ g/ml Ethidium Bromide

RNA gel loading buffer

4.4 μ l RNA sample
10 μ l Formamide
3.6 μ l Formaldehyde
2.0 μ l 10X MOPS buffer

Formamide, deionized

50 ml Formamide
5 g mixed bed ion exchange resin
(AG501-X8 BioRad)
Stir 30 minutes, filter through
Whatman No. 1 paper, store -20°C in aliquots

Lysis buffer

140 mM NaCl
1.5 mM $MgCl_2$
10 mM Tris, pH 8.5
0.5 % NP40
10 mM Vanadyl Ribonucleoside Complexes
(added before use)

2X PK buffer

200 mM Tris, pH 7.5
25 mM EDTA
300 mM NaCl
2 % SDS

Low salt buffer

10 mM Hepes, pH 7.5
0.05 % SDS
1.0 mM EDTA

10X MOPS electrophoresis buffer

400 mM MOPS, pH 7.0
100 mM Sodium acetate
10 mM EDTA

Northern blot hybridization buffer

0.5 M Sodium phosphate, pH 7.2
0.1 % BSA
3.5 % SDS
33 % Formamide
20 μ g/ml Yeast tRNA
1 mM EDTA

Appendix D. Sequencing Preparations.

Preparation of glass plates for sequencing gels:

Siliconization of short plate

0.2 ml Dimethyldichlorosilane

9.8 ml Chloroform

spread even film on one side of clean plate

bake for 3 hours at 80°C, wash

re-treat after 5 or 6 sequencing runs

Etching of long plate

5 M NaOH

coat one side of clean plate with NaOH, wash

Sequagel™ buffers for pouring gels

8% Acrylamide, 80 ml

25.6 ml Sequagel™ concentrate

46.4 ml Sequagel™ diluent

8.0 ml 10 X Sequagel™ buffer

0.5 ml 10 % (w/v) Ammonium persulfate

50 µl TEMED

10 X TBE (for 1 liter)

1 M Tris, pH 8.3 (121.2 g Tris base)

1 M Boric acid (61.7 g)

20 mM EDTA (7.4 g Disodium EDTA)

Sequencing gel fixer 10 % Methanol

10 % Acetic acid

Appendix E. Buffers for Protein Analysis

Protein Extraction Buffer

1.0 % Triton X100
0.1 % SDS
0.5 % DOC
0.1 M NaCl
10 mM Sodium phosphate, pH 7.4
1 mM PMSF
100 KIU Aprotinin

Sample loading buffer (2X)

20 % glycerol
4 % SDS
100 mM Tris, pH 6.8
0.02 % Bromphenol blue
4.25 % β Mercaptoethanol
(for reducing gels)

PAGE Buffer 10X

30.25 g Tris base
144.0 g Glycine
dist. H₂O to 1 liter

SDS PAGE Running Buffer

50 ml 10X PAGE buffer
2.5 ml 20% SDS solution
dist. H₂O to 500 ml

Electroblotting Buffer (3 liters)

30 ml 10X PAGE buffer
600 ml methanol
distilled H₂O to 3 liters

Immunoprecipitation Wash buffer

50 mM Tris pH 7.5
100 mM NaCl
1.0 % Triton X100
0.5 % DOC
0.1 % SDS
1 mM EDTA

BLOTTO for blocking Western Blots

2% Carnation Nonfat dry milk
1% Triton X100
50 mM Tris pH 7.5
10 mM EDTA

PAGE gel fixing and dehydration solution

500 ml Absolute Methanol
70 ml Glacial acetic acid
430 ml deionized water

Barth-Hepes Saline(for oocytes)

88 mM NaCl
1.0 mM NaHCO₃
0.82 mM MgSO₄
0.33 mM Ca(NO₃)₂
0.41 mM CaCl₂
10 mM Hepes
10 mg/liter penicillin and streptomycin
final pH 7.0 to 7.2 with NaOH, filter sterilized

Appendix F. List of Oligonucleotide Primers:

1. Human c-N-ras sequencing oligonucleotides:

<u>name</u>	<u>sequence, 5'to 3'</u>	<u>location in gene</u>
N-ras 12,13(5')	ATGACTGAGTACAACTGGT	Exon 1-5' (Clontech)
N-ras 12,13(3')	CTCTATGGTGGGATCATATT	Exon 1-3' (Clontech)
N-ras 61 (5')	CAAGTGGTTATAGATGGTGA	Exon 2-5' (Clontech)
N-ras 61 (3')	AGGAAGCCTTCGCCTGTCCT	Exon 2-3' (Clontech)
E3f	CGTTTTAGGAGCAGATTAAG	EXON 3-5'
E3r	AAGACCAGACAGGTATGGTA	EXON 3-3'
E4f	TTTATAGGGTGTGAAGATG	EXON 4-5'
E4r	TGCCATGTGTGGTGATGTAA	EXON 4-3'
E5f	ATACTTTTAAAGTTTTGTCA	EXON 5-5'
AP2 (AP04)	TTAGTACAATAATCTCTATT	EXON 5-middle, forward
CB41 (AP1)	CCTAGAAGTGGTTTGTAGAC	EXON 6-forward
Ci6	ATCTCCACTTTTTTCATAGG	EXON 6-middle, forward

2. MOUSE c-H-ras sequencing and PCR* oligonucleotides:

<u>name</u>	<u>sequence, 5'to 3'</u>	<u>location in cDNA</u>
CB63	CAGCCGCTGTAGAAGCTATG	CODON 12/13, forward
CB74*	AGGGACAGTTCATGACAGTC	3'UTR, reverse
α ex1	AAAGTGGTTCTGGATCAGCT	EXON 1, reverse
CB65-1	CGGCACCTCCATGTCCTGAG	3'UTR, reverse
25 α exon3	ACCATTGGCACATCATCTGA	EXON 3, reverse
5'PCR	GGTGAGGCAGGGACCAGCGA	INTRON E, forward
3'PCR	CCTGTGCCGGGACGGGCACA	INTRON E, reverse
5'intron D*	GCTATGGCATCCCCTACATT	EXON 3-middle, forward
3'intron D*	ATTTGCAGCTCATGCAGCCA	EXON 4, reverse
H5'1	AGTACATGCGCACAGGGGAG	EXON 2-middle, forward
H5'2*	AGTCCGTGAGATTCGGCAGC	EXON 4-middle, forward
5'UTR (AP55)	CTGTCATAGCTTCTACAGCG	EXON 1-beginning, reverse

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