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Advances in Brief

Specific Inhibition of K-*ras* Expression and Tumorigenicity of Lung Cancer Cells by Antisense RNA[1]

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Abstract

A human lung cancer cell line (H460a) with a homozygous spontaneous K-*ras* mutation was transfected with a recombinant plasmid that synthesizes a 2-kilobase genomic segment of the K-*ras* protooncogene in antisense orientation. Translation of the mutated K-*ras* mRNA in H460a cells was specifically inhibited, whereas expression of H-*ras* and N-*ras* was unchanged. A 3-fold growth inhibition occurred in H460a cells when expression of the mutated *ras* p21 protein was down-regulated by antisense RNA. However, cells remained viable despite the absence of K-*ras* expression. The growth of H460a tumors in *nu/nu* mice was substantially reduced by expressed K-*ras* antisense RNA.

Introduction

A wide spectrum of human cancers harbor *ras* genes activated by a single point mutation (1–12). Despite considerable knowledge of the structural aspects of the *ras* gene product, the functional role in physiological and pathological processes remains elusive (1). Cellular location and structural and biochemical similarities to G proteins suggest that *ras* gene products are involved in signal transduction (13, 14). We used an antisense RNA construct to block selectively the production of the mutated protein in the human non-small cell lung cancer (NSCLC) cell line NCI-H460A and examined the direct contribution of the mutated p21 protein to the malignant phenotype.

Materials and Methods

H460, H322, H226, and H522 NSCLC cell lines were generously provided by Drs. J. D. Minna and A. F. Gazdar (National Cancer Institute Naval Medical Oncology Branch, Bethesda, MD). All cell lines were grown in regular RPMI medium and 5% fetal calf serum in routine culture.

Plasmid Construction. A 2-kb genomic DNA fragment from the K-*ras* protooncogene was subcloned into an Apr-1-neo vector in both sense and antisense orientation. A 2-kb *Eco*RI/*Pst*I fragment containing second and third exon sequences together with adjoining flanking intron sequences was isolated from the SP6 vector (Oncogene Sciences) and Klenow enzyme was used to make blunt ends. Apr-1-neo vector was digested with *Bam*HI and blunt end ligation was performed to obtain the Apr-1-neo-AS or Apr-1-neo-S constructs.

DNA Transfections. H460a or H322a cells were electroporated with 10 µg of Apr-1-neo-AS or Apr-1-neo-S plasmid DNA. Forty-eight h after transfection G418 was added into the medium at a concentration of 300 µg/ml for H460a and 200 µg/ml for H322a. Individual colonies were picked up and grown in culture for further analysis.

Southern Blot Analysis. High molecular weight DNA was isolated, digested with *Eco*RI (Boehringer-Mannheim) (20 µg), electrophoresed in 0.8% agarose gel, transferred onto a Gene Screen membrane (NEN), and hybridized with a ^{32}P-nick-translated 2-kilobase genomic K-*ras* DNA probe.

Measurement of RNA Expression. Total cellular RNA was isolated from the cell lines (18). Twenty µg of total RNA was size fractionated in 4-morpholinepropanesulfonic acid/formaldehyde gel, transferred onto a Gene Screen membrane, and processed for hybridization with riboprobes. A 302-base pair genomic DNA of the K-*ras* gene was amplified by PCR spanning the third exon and intron sequences and was subcloned into a Bluescript vector. *In vitro* S and AS RNA probes were synthesized using either a T7 or T3 promotor.

Polymerase Chain Reaction. Polymerase chain reactions were performed as previously described using *Taq*I DNA polymerase (16). Oligonucleotide primers corresponding to the 5′ and 3′ regions of codons 12 and 61 of human K-*ras*, H-*ras*, and N-*ras* genes were synthesized. Two µg of genomic DNA was subjected to 35 cycles of amplification. DNA sequences of oligonucleotide primers used for PCR amplification are listed in Table 1.

Slot Blot Oligonucleotide Hybridization. PCR-amplified DNA samples (12.5, 25, and 50 ng) were blotted onto a Gene Screen membrane using a slot blot apparatus (Schleicher and Schuell). The filters were prehybridized and hybridized at 55°C in 6× standard saline citrate, 5× Denhardt's solution, and 100 µg/ml of salmon sperm DNA for 2 h. Filters were washed twice in 6× SSPE at room temperature and once for 30 min at 58°C. Finally, blots were washed for 5 min at 64°C. The filters were exposed to X-ray film for 12–24 h at −80°C.

Direct Sequencing of PCR-amplified DNAs. PCR DNA corresponding to the second exon was purified in 8% polyacrylamide gel. A single DNA band was excised and purified DNA was used for asymmetric amplification in 100 µl of PCR reaction mixtue. One (KA 61) amplimer was added to this mixture. After 20 cycles, single-stranded DNA was purified through gene clean (Bio 101) and DNA was eluted in 15 µl of water. Four µl of DNA was mixed with 4 µl of 10× *Taq*I buffer and 1 µl (10 pmol) of a second amplimer (KB 61) was used as a sequencing primer. DNA was sequenced using a Sequenase kit.

RNA PCR Analysis. cDNA synthesis was carried out in a total volume of 20 µl containing 5 µg of total RNA and oligo(dT) as a primer (17). A portion of the cDNA corresponding to the first and second exons was amplified to monitor the level of endogenous K-*ras* mRNA (Fig. 2*A*) using KA12 and KB61 amplimers. Denaturation, annealing, and extension were done at 92°C for 1 min, 51°C for 1 min, and 74°C for 1 min, respectively. However, annealing temperatures for N-*ras* and H-*ras* were 44°C and 42°C, respectively. In addition, two amplimers were also used in the same reaction mixture to amplify a 118-base pair fragment of the p53 gene as an internal control. PCR products were either transferred onto a membrane and hybridized with ^{32}P-labeled cDNA probe or, alternatively, they were directly labeled during the last cycle of amplification by adding 1 µCi of [^{32}P]dCTP. The labeled PCR products were loaded on an 8% nondenaturing polyacrylamide gel. The gel was photographed after ethidium bormide staining, dried, and exposed to X-ray film overnight at −80°C.

Western Blot Analysis of *ras* Protein. Protein extracts were prepared by lysing cells in TBS (10 mM Tris, pH 7.5-100 mM NaCl-1 mM

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; PCR, polymerase chain reactions; cDNA, complementary DNA; AS, antisense orientation; S, sense orientation.

Table 1 *DNA sequences of oligonucleotide primers used for PCR amplification*

Primers	Sequence	Target
KA61	5′ TTC CTA CAG GAA GCA AGT AGT A 3′	K-*ras* 2nd exon
KB61	5′ ACA CAA AGA AAG CCC DCC CCA 3′	
KA12	5′ GAC TGA ATA TAA SCT TGT GG 3′	K-*ras* 1st and 2nd exon
KB61	5′ ACA CAA AGA AAG CCC DCC CCA 3′	
HA12	5′ GAC GGA ATA TAA GCT GGT GG 3′	H-*ras* 1st and 2nd exon
HB61	5′ CGC ATG TAC TGG TCC CGC AT 3′	
NA12	5′ GSC TGA GTA CAA ACT GGT GG 3′	N-*ras* 1st and 2nd exon
NB61	5′ ATA CAC AGA GGA AGC CTT CG 3′	

phenylmethylsulfonyl fluoride-1% Nonidet P-40–1% deoxycholate). The extracts were cleaned by centrifugation at 10,000 × *g* for 1 h. The protein concentration of the supernatant was calculated spectrophotometrically. Protein, 500 µg, was size fractionated in 12.55% sodium dodecyl sulfate-polyacrylamide gel and electroblotted onto nitrocellulose membranes. *ras*-specific p21 protein was detected using either K-*ras*- or pan *ras*-specific monoclonal antibody (Oncogene Sciences) followed by ^{125}I-labeled goat anti-mouse second antibody.

Tumorigenicity in Nude Mice. The tumorigenicity of these cell lines was examined by s.c. inoculation of 10^5 (Fig. 3*B*) and 10^6 cells in *nu/nu* mice. Each cell line was injected into 5 animals. Tumors were measured with linear calipers in 2 orthogonal directions by the same observer.

Results and Discussion

Segments of the K-*ras* gene containing first and second exons were amplified from a number of NSCLC cell line DNAs by polymerase chain reaction (16) and subsequently hybridized with a set of ^{32}P-labeled oligonucleotide probes (Fig. 1, *A1* and *A2*). Mutations were confirmed by a direct PCR DNA-sequencing method. A homozygous mutation at codon 61 was detected in the NCI-H460A large cell undifferentiated NSCLC cell line with a normal glutamine residue (CAA) substituted by histidine (CAT). This cell line is highly tumorigenic in nude mice.

A recombinant plasmid clone was constructed using a wild-type 2-kilobase K-*ras* genomic DNA segment carrying second and third exons together with flanking intron sequences subcloned into an Apr-1-neo expression vector (18) in the AS orientation. S plasmid constructs were used as a control (Fig. 1*B*). AS or S K-*ras* RNA synthesis was accomplished by transfecting H460a cells, a cloned derivative of the NCI-H460A cell line, with Apr-1-neo-AS or Apr-1-neo-S constructs by electroporation. The β-actin promoter of the vector was constitutively capable of directing the synthesis of RNA from the inserted DNA. The Apr-1-neo vector offered suitable G418 marker gene expression for selection of the transfectants. Individual G418-resistant colonies were selected and grown in culture for further analysis. Stable integration of the plasmid DNA in the transfectants was examined by Southern hybridization with a 2-kilobase DNA insert from the original plasmid clone as a probe (Fig. 1*C*). The Southern blot analysis showed a single 3-kilobase *Eco*RI band corresponding to the endogenous K-*ras* gene in the parental H460a cell line, but additional bands were observed in the individual clones indicating single or multiple copy inserts.

The extent of stable AS RNA expression and its effect on the endogenous K-*ras* mRNA level was investigated. Total RNA was extracted from subconfluent, growing cultures (18). The presence of AS and S RNA was detected by Northern blot hybridization using either an S or AS RNA probe synthesized *in vitro* from a Bluescript vector carrying a 302-base pair K-*ras* DNA insert corresponding to the third exon and part of the intron sequences (Fig. 1*D*). Interestingly, the clones carrying the Apr-1-neo-AS vector show one RNA band at about 1.5 kilobases, but the cells carrying the S construct show two RNA species. The reason for this is unknown, but the possibility exists that the RNA synthesized from the genomic DNA under control of the β-actin promoter could be processed *in vivo*. However, no corresponding hybridization band was detected in H460a cells, which indicated that a significantly higher level of K-*ras* RNA was synthesized under the β-actin promoter.

We next analyzed the p21 protein level in these transfectants by Western blot analysis (Fig. 1, *E* and *F*). A K-*ras*-specific p21 monoclonal antibody (Oncogene Science) was used to determine the level of K-*ras* protein in transfectants, parental H460a cells, and Calu-1 cells, which have a high level of K-*ras* gene expression (Fig. 1*E*). Western blot analysis showed a 95% reduction in K-*ras* p21 protein synthesis in the clones expressing the AS RNA, while parental cells, S K-*ras* clones, and Calu-1 cells showed a significant level of K-*ras* p21 protein. These results indicate that AS RNA can effectively block the synthesis of K-*ras*-specific protein. Since members of the *ras* gene family share a great deal of sequence homology and code for a similar p21 *ras* protein, we examined the total *ras* protein product in these clones using a pan *ras* monoclonal antibody (New England Nuclear) to determine whether a reduced level of K-*ras* protein reflects any change in H-*ras* and N-*ras* p21 protein synthesis (Fig. 1*F*). Western blot analysis revealed only a slight decrease in overall *ras* protein level in all clones containing Apr-1-neo-AS as compared to 460a parental cells.

The effect of AS RNA on the specific production of mature endogenous K-*ras* mRNA was analyzed by cDNA PCR (Fig. 2). cDNA synthesized from the total RNA (18) was subjected to PCR amplification using amplimers corresponding to the 5′ end of the first exon and the 3′ end of the second exon (Fig. 2*A*). Because the AS RNA was generated only from a second and third exon of the K-*ras* gene, PCR-amplified cDNA represented the level of endogenous K-*ras* mRNA. A 246-base pair amplified DNA fragment was labeled by [^{32}P]dCTP and subsequently analyzed by polyacrylamide gel electrophoresis. In addition, a 118-base pair segment of endogenous p53 cDNA was coamplified in the same reaction mixture using p53-specific amplimers to serve as an internal control for the PCR.

Results showed that H460a cells, clones expressing S RNA, and the Calu-1 cell line expressed K-*ras* mRNA, as evidenced by the presence of a high level of amplification of the 246-base pair cDNA product (Fig. 2*B*). H460a clones expressing AS RNA showed very little amplification, and cellular K-*ras* mRNA synthesis appeared to be completely inhibited (Fig. 2*B*, *lanes 5 and 6*). In contrast, the endogenous p53 expression remained unaffected. This prompted us to investigate the level of expression for other *ras* genes in these clones. We used the same cDNA PCR methodology to analyze the N-*ras* and H-*ras* mRNA level using N-*ras*- and H-*ras*-specific oligonucleotides as amplimers. A steady state level of H-*ras* and N-*ras* gene expression was observed, but no obvious change in either Apr-1-neo-AS or Apr-1-neo-S transfectants was noticed (Fig. 2, *C* and *D*). The p53 gene expression serving as a control in these experiments remained unaffected. Thus, inhibition of K-*ras* expression by our AS RNA construct is specific.

H460a clones expressing AS K-*ras* RNA continued to grow in culture. However, H460a Apr-1-neo-AS transfectants showed a 3-fold reduction in growth, compared to the H460a Apr-1-neo-S transfectants and the parental H460a cells (Fig. 3*a*). The H322 NSCLC line has wild-type *ras* family genes. H322 Apr-1-neo-AS and Apr-1-neo-S transfectants had identical growth characteristics, indicating that inhibition of wild-

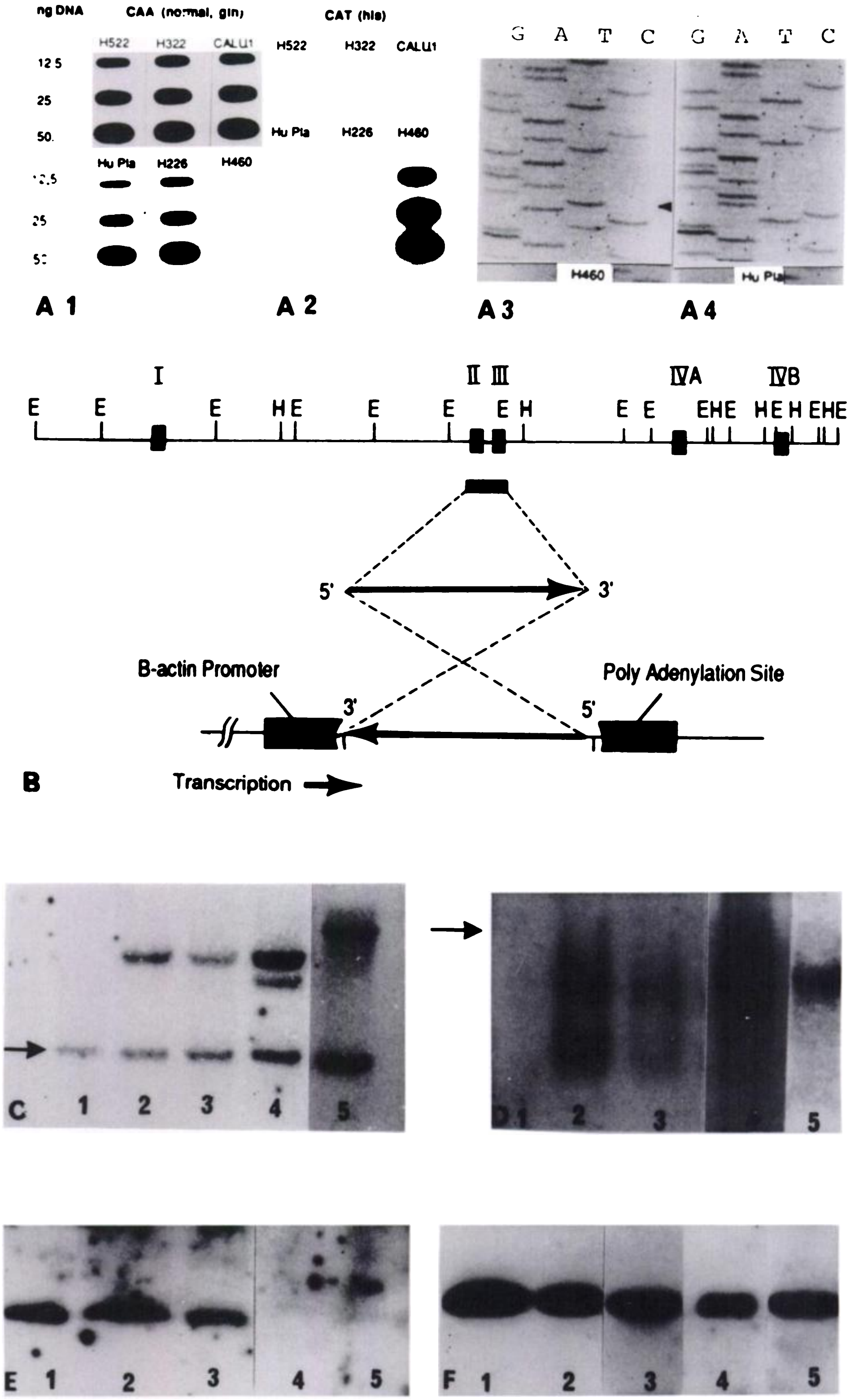

Fig. 1. In *A*, the second exon of the K-*ras* gene was amplified from genomic DNA of H522, H322, Calu-1, H226, H460a, and human placenta by PCR, blotted onto a Gene Screen membranes, and hybridized with ^{32}P-end-labeled oligonucleotide probes. *A1*, presence of wild-type glutamine residue (CAA) at 61 codon in five cell lines except H460a. The same blot was reprobed with a histidine-specific mutated oligo probe (CAT) and only the H460a cell line PCR DNA hybridized (*A2*). The mutation was confirmed by direct PCR DNA sequencing. Wild-type K-*ras* 61 codon sequence in human placenta (*A3*) was compared with the H460a cell line (*A4*). In *B*, a 2-kilobase genomic DNA from the K-*ras* oncogene was subcloned into an Apr-1-neo vector in both sense and antisense orientation. A 2-kilobase *Eco*RI/*Pst*I fragment containing second and third exon sequences together with adjoining flanking intron sequences was isolated from the SP6 vector (Oncogene Sciences) and Klenow enzyme was used to make blunt ends. Apr-1-neo vector was digested with *Bam*HI and blunt end ligation was performed to obtain the Apr-1-neo-AS or Apr-1-neo A constructs. *C*, a Southern blot analysis of the K-*ras* oncogene in H460a and H460a transfectants. Blots were probed with a ^{32}P-nick-translated 2-kilobase *Eco*RI/*Pst*I insert DNA. *Lane 1*, H460a; *lanes 2* and *3*, H460a transfected with Apr-1-neo-S C1#1 and C2#1; *lanes 4* and *5*, H460a cells transfected with Apr-1-neo-AS, C3#32, and C2#32, respectively. *D*, a Northern blot analysis of sense and antisense K-*ras* RNA. *Lane 1*, H460a; *lanes 2* and *3*, Apr-1-neo-S transfectants; *lanes 4* and *5*, Apr-1-neo-AS transfected clones. *E* and *F*, Western blot analysis of K-*ras*-specific p21-protein (*1E*) and total *ras* protein (*1F*) was done using either pan *ras* or K-*ras*-specific monoclonal antibodies. *Lane 1*, Calu-1 control cell line overexpressing K-*ras*-specific protein; *lane 2*, H460a; *lane 3*, H460a Apr-1-neo-S; *lanes 4* and *5*, H460a Apr-1-neo-AS.

type K-*ras* is not sufficient to alter the tumor cell growth rate (data not shown). These results together indicate that the presence of sense K-*ras* RNA did not alter the growth kinetics of H460a cells. However, the marked growth retardation of the K-*ras* Apr-1-neo-AS transfectants suggests that the mutated p21 protein contributes to the faster growth rate of these cells.

The tumorigenicity of cell lines expressing AS RNA was assessed by s.c. injection of 10^5 and 10^6 cells in *nu/nu* mice. Inoculation of H460a cells at both doses led to the formation of tumors in 15 days in all mice (3–5 mice/group in 3 separate experiments). No tumor developed in mice treated with 10^5 cells for both clones of H460a AS cells during 120 days of observation in a total of 10 mice, whereas all mice receiving H460a cells developed tumors (data not shown). When the inoculum was increased to 10^6 cells, tumors grew in all mice, but the tumors in mice receiving AS clones grew at a slower

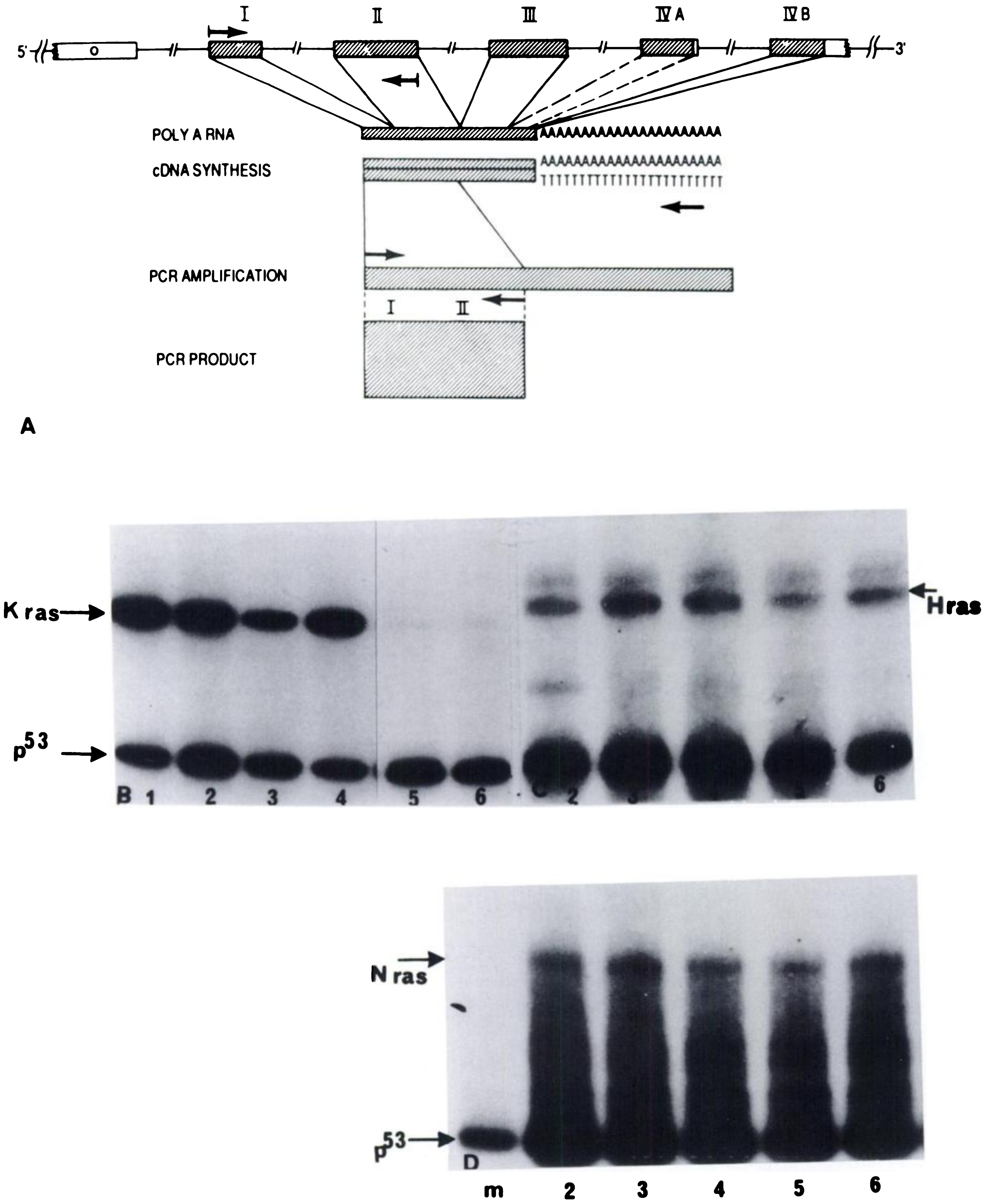

Fig. 2. *A*, schematic diagram of K-*ras* RNA synthesis. A segment of *ras* cDNA was amplified using oligonucleotide primers corresponding to the 5' region of the first exon and 3' of the second exon (*arrows*) for RNA PCR analysis. *B*, a RNA PCR analysis done to compare the level of K-*ras* message in H460a and H460a transfectants. As a control, a portion of p53 gene was coamplified with the p53-specific primer which served as an internal contol. *C* and *D*, H-*ras*- and N-*ras*-specific amplimers used to quantitate H-*ras*/N-*ras* RNA in the transfectants and parental cell lines. p53 gene amplification is shown as an internal control. *Lane 1*, Calu 1; *lane 2*, H460a; *lanes 3 and 4*, H460a transfected with Apr-1-neo-S, C1 #1 and C2 #1; *lanes 5 and 6*, H460a cells transfected with Apr-1-neo-AS, C3 #32 and C2 #32.

rate than H460a cells or the S control (Fig. 3*B*). Tumors were excised and analyzed for K-*ras* expression by cDNA-PCR. K-*ras* expression was not detected in tumors arising from injection of AS clones but was present in S clones and H460a tumors.

The above experiments indicate that, in H460a cells engineered to synthesize AS K-*ras* RNA, the levels of K-*ras* mRNA and K-*ras* p21 protein are effectively down-regulated. Reduction in the expression of K-*ras* mutated gene reproducibly reduced the rate of tumor growth in *nu*/*nu* mice. Our studies show that a construct can be made that distinguishes among members of

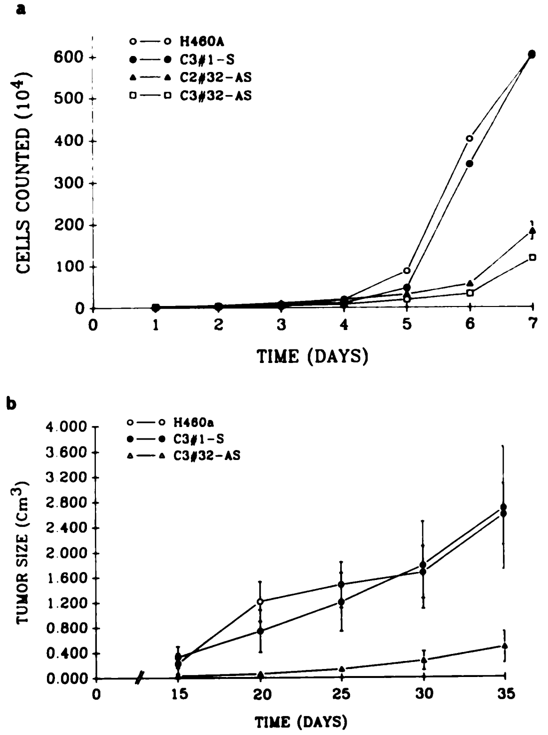

Fig. 3. *a*, *in vitro* growth curve. Cells were seeded at 10^4 cells/plate and grown for a 7 days. Cells were harvested and counted in a hemocytometer at 24 h intervals. Growth curves for H460a and H460a cells transfected with the Apr-1-neo-S vector do not show any significant difference, but H460a transfectants carrying Apr-1-neo-AS showed growth inhibition (*b*). H460a cells (10^6) were injected s.c. into the left flanks of female Balb/c *nu/nu* mice. Cross-sectional diameters of the external tumor were measured without knowledge of the cell group. Tumor volume was calculated by assuming a spherical shape with the average tumor diameter calculated as the square root of the product of cross-sectional diameters. Palpable tumors were first detected on day 15. *Point*, mean; *bar*, ±SE. C3#32-AS, $n = 5$; C3#1-S, $n = 5$; H460a, $n = 3$. C3#32-AS compared to C3#1-S or H460a on days 20, 25, 30, 35 ($P < 0.05$ by Wilcoxon's test).

the *ras* family. Previous studies with AS oligonucleotides showed inhibition of p21 expression which led to cell death (19, 20). Our data indicate that AS RNA generated from the genomic DNA of the K-*ras* gene can specifically inhibit K-*ras* expression. In our model inhibition of activated K-*ras* reduced the growth rate of the H460a cells. However, there was no affect on cell viability or continued growth in culture. This suggests that redundancy in p21 expression may compensate for absence of expression by one member of this family so that functions essential for maintenance of cell viability are preserved. However, tumorigenicity was maintained in the absence of activated K-*ras* expression, although the rate of tumor growth was diminished. We hypothesize that, in human NSCLC, *ras* mutations confer a growth advantage to the malignant cell.

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