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Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line

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We have carried out an analysis of amplified DNA sequences present in a tumorigenic mouse cell line, designated 3T3DM, to determine if the presence of cellular transforming activity is correlated with the elevated expression of any of the amplified genes. These studies utilized a selection protocol that allowed for DNA sequence amplification after the introduction of each gene into non-transformed recipient cells. Cell lines obtained from this selection protocol were assayed for tumorigenicity in nude mice. The results provided evidence that a gene, *mdm2*, that is amplified more than 50-fold in the 3T3DM cell line, induces tumorigenicity when experimentally overexpressed in NIH3T3 cells and in Rat2 cells. Analysis of the predicted amino acid composition of the *mdm2* product(s) revealed features similar to those that have been shown to be functionally significant in certain DNA binding proteins/transcriptional activators. These include two potential metal binding motifs and a negatively charged domain rich in acidic amino acid residues. Overall, the data support the conclusion that *mdm2* represents an evolutionarily conserved gene with tumorigenic potential and a predicted role in mechanisms of cellular growth control.

Key words: amplification/double minutes/overexpression/oncogenes/tumorigenicity

Introduction

The identification of cellular genes directly involved in pathways of growth control and differentiation has often derived from investigations of cancer cells in which such genes are genetically altered. Enhanced expression, consequent to a process of DNA amplification, is a relatively frequent mechanism by which a number of growth control genes can be converted to oncogenic forms. These include the cellular genes c-*myc*, c-*myb*, N-*myc*, L-*myc*, c-*Ki-ras*, c-*abl*, c-*erbB2*, and others (Alitalo and Schwab, 1986; Schwab and Amler, 1990). A functional analysis of amplified DNA in tumor cells, then, should allow the isolation of other genes that have a central role in processes of normal cellular proliferation and oncogenic transformation. A candidate for such a gene is described in this report.

The tumor cell line used for this study, 3T3DM, contains amplified DNA sequences (George and Powers, 1982; Cahilly-Snyder *et al.*, 1987) in the form of double minutes (DMs), which are small, acentromeric, extrachromosomal nuclear bodies (Hamlin *et al.*, 1984; Schimke, 1984; Stark and Wahl, 1984). The DMs have been stably maintained during long-term growth of the 3T3DM cells *in vitro* and *in vivo*. This pattern would be expected if the amplified domain includes a gene that, as a consequence of its elevated expression, provides these cells with some growth advantage; this advantage is most likely related to their tumorigenic properties. Our initial analyses of these cells, however, revealed that no known oncogene or growth factor-encoding gene is amplified (Cahilly-Snyder *et al.*, 1987), raising the interesting possibility that the amplified domain includes a previously unrecognized growth-control gene. In the absence of apparent homology to known sequences, the isolation of such a gene required the development of a strategy based on: (i) The identification of transcribed sequences within the amplified domain and (ii) a functional test of candidate genes for evidence of transforming potential. The results of such an approach are described here. The data provide evidence that a gene, designated *mdm2*, that is amplified more than 50-fold in the 3T3DM cells, induces tumorigenicity when overexpressed in NIH3T3 cells and Rat2 cells.

Results

In work leading to the present study, we isolated and initially characterized three genes that are amplified and overexpressed in the 3T3DM cells. The isolation of two of these, *mdm1* and *mdm2*, by differential screening of a 3T3DM cDNA library has been reported (Cahilly-Snyder *et al.*, 1987). The *mdm1* and *mdm2* genes map to mouse chromosome 10, region C1-C3 (Cahilly-Snyder *et al.*, 1987), and sequences homologous to each of these genes are present on human chromosome 12 (Fakharzadeh *et al.*, unpublished data). A third amplified and overexpressed gene, *mdm3*, was subsequently identified and isolated using field inversion gel electrophoresis analysis, and chromosome walking from the closely linked *mdm2*. This approach was based on the idea that clusters of C+G residues ('CpG islands') can serve as flags along the DNA for transcribed units (Bird, 1987). Because all three of the amplified genes contain CpG islands at their 5′ ends (Snyder *et al.*, 1988; George *et al.*, unpublished data), we were able to construct a long-range map of the CpG clusters along the DM. Information from this map, coupled with other studies, allowed us to conclude that there is no evidence for the presence of other overexpressed genes on the amplified domain.

Isolation of cell lines overexpressing mdm transcripts

After the isolation and initial characterization of *mdm1*, *mdm2*, and *mdm3*, experiments were carried out to determine if the elevated expression of any of these genes could be correlated with the presence of cellular transforming activity. To do this, we used a selection protocol that allowed

for DNA sequence amplification after introduction of each gene into nontransformed recipient cells.

Characterization of a number of cDNA clones demonstrated that each of the amplified genes encodes multiple transcripts varying in coding capacity (Snyder *et al.*, 1988; George *et al.*, unpublished obvservations). Therefore, studies designed to examine transforming potential were carried out using full-length genomic equivalents of the genes, rather than with cDNA clones, in an attempt to reproduce faithfully their expression patterns. Each gene was cloned into pCV001, a cosmid vector that contains two dominant selectable markers: the bacterial *neo* gene and the mouse *DHFR* gene (Choo *et al.*, 1986). The pCV001 constructs containing either *mdm1*, *mdm2*, or *mdm3* were transfected into non-transformed mouse NIH3T3 cells or Rat2 cells. For each transfection, pooled G418-resistant clones were subjected to gradually increasing concentrations of methotrexate (MTX) to select for cells that had amplified the *DHFR* gene. It was expected, and subsequently confirmed, that the correspondingly linked *mdm* gene could be coamplified in this way. NIH3T3 cells and Rat2 cells that were transfected with the cosmid vector alone and similarly subjected to both G418 and MTX selection served as additional controls.

Pools of cells overexpressing appropriately sized transcripts of *mdm1*, *mdm2* or *mdm3* were obtained from this protocol. A representative example of the RNA levels achieved in various NIH3T3 transfectants is presented in Figure 1. For the Rat2 transfectants, similar results were obtained (data not shown). For discussion purposes, the pools of NIH3T3 transfectants containing the *mdm1*, *mdm2*, *mdm3*, or vector only constructs will be referred to as N/mdm1, N/mdm2, N/mdm3 and N/pCV001, respectively. Similarly, the Rat2 transfectants will be referred to as R/mdm1, R/mdm2, R/mdm3, and R/pCV001, respectively.

Analysis of tumorigenicity

Cell lines resulting from this selection scheme were tested for tumorigenicity in athymic (nude) mice by subcutaneous injection. The results demonstrated that a series of *mdm2*-overexpressing NIH3T3 transfectants (N/mdm2) had acquired tumorigenic properties (Table I). In contrast, the N/mdm1, N/mdm3, and N/pCV001 transfectants failed to form tumors under the same conditions (the one N/mdm1 tumor observed did not overexpress *mdm1* and probably resulted from a spontaneous mutational event). The data summarized in Table I were generated using three independently generated pools of N/mdm2 transfectants, and two independently derived pools of N/mdm1, N/mdm3 and N/pCV001 transfectants.

In more limited trials, the same results were obtained with the Rat2 transfectants: an *mdm2*-overexpressing cell line (R/mdm2) was found to be tumorigenic, whereas the R/mdm1, R/mdm3 and R/pCV001 cell lines were negative for tumor formation (Table I). Although one athymic mouse injected with R/pCV001 control cells developed tumors, this finding was not reproducible. In six other tests of R/pCV001 cells, no tumors developed in any of the injected mice. Thus, as expected, Rat2 cells containing amplified copies of only vector sequences are not tumorigenic. Taken together, the *mdm2*-overexpressing transfectants produced tumors in 16/16 trials, whereas for all of the other cell lines combined, tumors appeared in only 3/38 tests.

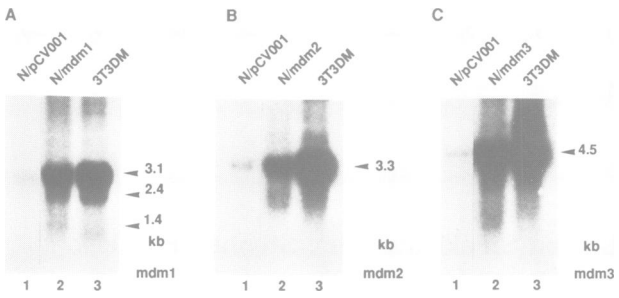

Fig. 1. Analysis of RNA from NIH3T3 transfectants. Each lane contains 10 μg of total RNA isolated from the cell lines indicated. The RNA blots were hybridized to the following ^{32}P-labeled probes: (**A**) *mdm1* cDNA probe p12; (**B**) *mdm2*cDNA 11B; (**C**) *mdm3* cDNA probe pB1.5. The various pools of NIH3T3 transfectants were first selected for resistance to G418 and then for resistance to MTX (final concentration 0.4 μM).

Table I. Tumorigenicity testing of transfectants

Cell line	Tumorigenicity*	Cell line	Tumorigenicity*
N/mdm2	12/12**	R/mdm2	4/4
N/mdm1	1/8	R/mdm1	0/4
N/mdm3	0/8	R/mdm3	0/2
N/pCV001	0/8	R/pCV001	2/8

Cell lines were injected subcutaneously into nude mice at a dose of 1×10^6 cells or 5×10^6 cells/injection and monitored over a period of 12–15 weeks.
*Tumorigenicity defined as number of tumors/number of injections.
**10 of the 12 tumors observed, including all (6/6) of the tumors resulting from the higher dosage injections, developed at about five weeks (average); two of the tumors, resulting from inoculations of 1×10^6 cells, appeared at 11 weeks.

RNA blot analysis confirmed that cells derived from the N/mdm2-induced tumors and the R/mdm2-induced tumors continue to overexpress *mdm2*. Figure 2 illustrates the relative abundance of *mdm2* transcripts in RNA samples prepared directly from two primary N/mdm2 tumors (lanes 3 and 4) and from two R/mdm2 tumors (lanes 7 and 8). In each case, *mdm2* RNA levels are about 10–15-fold higher than those of the NIH3T3 cells, based on RNA dilution assays. Similar results were obtained with RNA samples isolated from cell lines established from several other N/mdm2 tumors. Southern blot analysis demonstrated that the degree of *mdm2* amplification in these cells directly correlates with the degree of overexpression (data not shown). The elevated *mdm2* expression appears to represent a stable phenotype; one of the N/mdm2 cell lines has now been maintained in culture for six months in the absence of MTX selection and there has been no detectable reduction in the abundance of *mdm2* transcripts (unpublished data).

Overall, these results strongly indicate that enhanced expression activates an oncogenic potential of the *mdm2* gene and implicates this gene as one functioning in pathways of cellular growth control.

Evolutionary conservation of mdm2 sequence

DNA blot analysis was carried out to examine the extent to which the *mdm2* sequence has been conserved in evolution. DNA samples isolated from cells of several eukaryotic organisms, including human, Chinese hamster, rabbit and

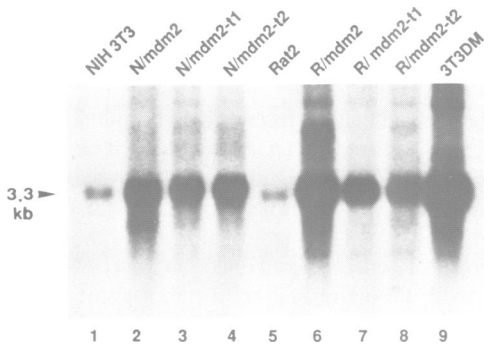

Fig. 2. RNA blot analysis illustrating relative abundance of *mdm2* transcripts in two N/mdm2-induced tumors (N/mdm2-t1, lane 3; N/mdm2-t2, lane 4) and in two R/mdm2-induced tumors (R/mdm2-t1, lane 7; R/mdm2-t2, lane 8) compared to the abundance in control cells (NIH3T3, lane 1; Rat2, lane 5) and in pools of transfectants that were analyzed for tumorigenicity (N/mdm2, lane 2; R/mdm2, lane 6). Each lane contains 10 μg total RNA.

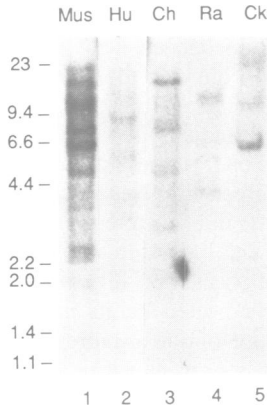

Fig. 3. DNA blot analysis illustrating the presence of *mdm2*-related sequences in DNA samples (5 μg) isolated from the following sources: mouse (Mus); human (Hu); Chinese hamster (Ch); rabbit (Ra); chicken (Ck). The 1.3 kb *mdm2* cDNA probe encompasses nucleotides 261–1605 in Figure 5. Final filter washing conditions were 0.2 × SSC, 60°C. Positions of molecular weight markers (kb) are indicated at the left side of the figure.

chicken, contain sequences that hybridize well to an *mdm2* cDNA probe (Figure 3). Bands of hybridization are also evident in DNA isolated from monkey, frog, yeast and sea urchin (data not shown). In addition, *mdm2* is expressed in a broad range of embryonic and adult mouse tissues; examples are presented in Figure 4. These results support the conclusion that *mdm2* is an evolutionarily conserved gene that provides a fundamental cellular function.

Nucleotide sequence analysis

Clues to the possible mechanism of action of *mdm2* were sought in its sequence composition. Figure 5 depicts the nucleotide coding region and the amino acid sequence predicted for the longest open reading frame determined for this gene. The figure is based on the analysis of genomic clones and at least eight different, overlapping cDNA clones isolated from two 3T3DM cDNA libraries. The largest cDNA insert, ~3 kb in size, includes the entire open reading frame illustrated in Figure 5. The analysis of cDNA clones also revealed that *mdm2* can generate a number of distinct mRNA transcripts that differ internally and at their 5′ ends (see legend to Figure 5). An ATG codon (encoding amino

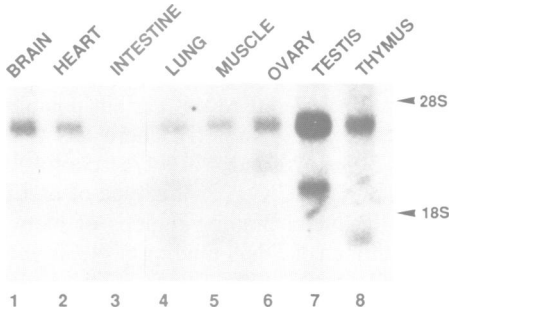

Fig. 4. RNA blot analysis illustrating the presence of *mdm2* transcripts in a number of tissues obtained from adult mice. Each lane contains 5 μg total RNA. The presence of equivalent amounts of RNA in each lane was confirmed by ethidium bromide staining (not shown). Positions of 28S and 18S rRNA are indicated to the right of the figure.

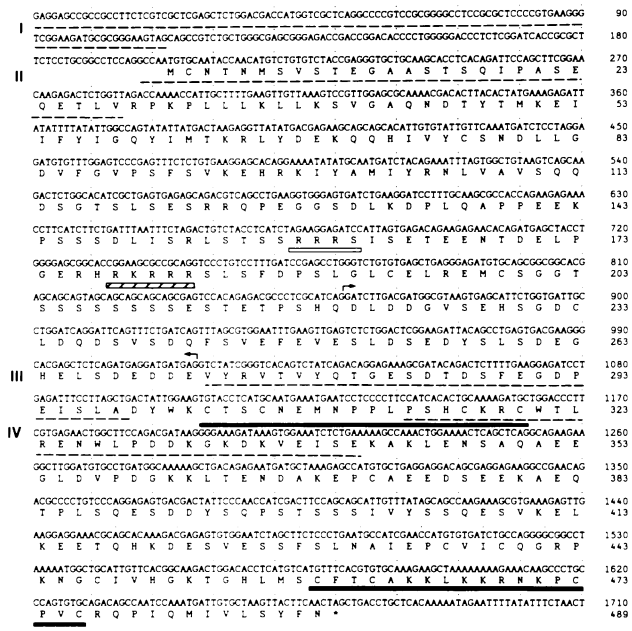

Fig. 5. Nucleotide and predicted amino acid sequence of *mdm2*. The sequences shown were derived from the analysis of genomic DNA and eight overlapping cDNA clones. Regions designated I–IV, indicated by a dashed underline (----) represent sequences that, presumably because of different promoter utilization or alternative splicing, were excluded from some of the cDNA clones. Of the eight cDNAs examined, region I was present in one clone, region II was excluded from two clones, and regions III and IV were each excluded from one clone. Other highlighted domains include a region (amino acid residues 221–272) rich in acidic amino acids (↱ ↰), a potential site of phosphorylation by protein kinase A, RRRS (▭), a potential nuclear localization signal, RKRRR (▨), and two zinc finger-like elements (solid underline).

acid residue 1) was chosen as a likely translational start site, because this represents the first in-frame ATG located downstream of a series of transcriptional start sites mapped using RNase protection- and primer extension assays (George *et al.*, unpublished data). The long open reading frame shown in Figure 5 could direct the synthesis of a 489 amino acid polypeptide, with a calculated molecular mass of 54 kd. Clearly, however, a number of distinct products could be encoded by this gene, and further analyses are required to fully characterize its complex expression pattern and the relationship of the various transcripts to the observed oncogenicity.

Although a search of nucleotide and protein sequence databases did not detect significant homologies to previously reported genes, examination of the predicted amino acid composition of *mdm2* did reveal certain notable features. They include the following: (i) There are two domains (amino acids 303–320 and 459–476) conforming to the pattern $CX_{2-4}CX_{2-15}CX_{2-4}C$; this type of motif has been described in the metal binding regions of many proteins, including a number of DNA binding proteins (Berg, 1986; Evans and Hollenberg, 1988). (ii) An acidic amino acid-rich domain (within 52 residues encompassing amino acids 221–272) is located just upstream of the first zinc finger-like motif. This region includes a high percentage (42%) of glutamic acid and aspartic acid residues and has a calculated net negative charge of −20. This segment resembles the acidic activation domains present in some transcriptional activators (Ptashne, 1988; Sigler, 1988). (iii) A region of basic amino acids (RKRRR) at residues 178–182 may serve as a nuclear localization signal (Dingwall and Kaskey, 1986). (iv) Several potential phosphorylation sites are present, including a potential protein kinase A recognition site (RRXS) at amino acid positions 158–161 (Kemp and Pearson, 1990). Given the overall features of the predicted *mdm2* product, it is intriguing to speculate that this gene encodes a potential DNA binding protein that functions in the modulation of expression of other genes and, when present in excess, interferes with normal constraints on cell growth.

Discussion

Investigations on a variety of primary tumor cells and tumor cell lines have revealed the presence of amplified copies of genes with known or suspected roles in normal cellular proliferation and the development of cancer. Therefore, other tumors exhibiting cytological hallmarks of gene amplification, such as DMs, represent a potential resource for identifying previously unrecognized genes with growth-associated properties. With this in mind, we carried out a functional analysis of amplified DNA sequences in a DM-containing, tumorigenic mouse cell line (3T3DM). As reported here, this work has led to the identification of an evolutionary conserved gene with oncogenic potential and a predicted role in mechanisms of growth control. The latter conclusion is supported by the fact that acentromeric DMs seem to persist in mammalian cells only if their presence provides some selective advantage (Hamlin *et al.*, 1984; Schimke, 1984; Stark and Wahl, 1984). We suggest, therefore, that the naturally occurring gene amplification event in the 3T3DM cells has served to unmask the *mdm2* gene as one important in certain cell proliferation events.

The tumorigenic N/mdm2 and R/mdm2 transfectants derived in this study exhibit a 10- to 15-fold amplification and overexpression of *mdm2*. In comparison, this gene is amplified and overexpressed about 50–60-fold in the 3T3DM cells, which give rise to rapidly growing tumors within 1–2 weeks after subcutaneous injection into athymic mice. The more rapid appearance of 3T3DM-induced tumors, relative to the N/mdm2- and R/mdm2-induced tumors (see Table I), may be related to the much higher level of *mdm2* amplification in the 3T3DM cells. Alternatively, 3T3DM cells may possess other genetic lesions that contribute to their more aggressive tumorigenicity. It will be of interest to determine if a higher level of expression of specific *mdm2* cDNAs can be achieved in appropriate recipient cells, and if this will result in a more rapid appearance of tumors.

We have noted that the tumorigenic N/mdm2 transfectants do not grow well in soft agar, and show little or no focus-forming ability (unpublished data). This finding was not unexpected, since these properties are also displayed by the 3T3DM cells. It is clear from studies on other oncogenes/growth control genes assayed under a variety of experimental conditions that a manifestation of tumorigenic potential can be dissociated from other parameters used to assess cell transformation. It has been demonstrated, for example, that N-*myc* (Small *et al.*, 1987), c-*myc*, and the adenovirus *E1a* gene (Kelekar and Cole, 1986) can oncogenically transform established or previously immortalized rodent fibroblasts with only limited effects on cell morphology and/or anchorage-independent growth. Elucidating the molecular pathway through which *mdm2* normally functions and the mechanisms by which excess product transforms cells may be aided by exploring this gene's interactions with other growth regulators/oncogenes.

As illustrated in Figure 5, the *mdm2* gene can give rise to alternatively processed transcripts differing in coding capacity; additional studies will be needed to clarify whether one, or more than one, of these products is responsible for the observed tumorigenicity. Analysis of the predicted amino acid sequence of the mdm2 protein(s) revealed features that are similar to those described in a number of DNA binding proteins/transcription factors. If *mdm2* does encode such a factor, it might be expected that its altered expression could disrupt the regulation of one or more genes needed to maintain normal constraints on cellular proliferation. Experiments are in progress to address that question, and to further assess the role of this new genetic marker in pathways of growth control.

Materials and methods

Cell lines

The 3T3DM cell line is a spontaneously transformed, tumorigenic derivative of a Balb/c line and has been previously described (George and Powers, 1982; Cahilly-Snyder *et al.*, 1987). Nontransformed recipient cells used in the DNA transfection protocols were NIH3T3 cells (a gift of J.Gibbs), and Rat 2 cells (American Type Culture Collection, CRL1764). All cell lines were routinely maintained in MEM supplemented with 7.5% fetal bovine serum and antibiotics.

Molecular clones

The cosmid vector pCV001 (a gift of Y.-F.Lau and Y.W.Kan) carries expression units for the bacterial neomycin-resistance gene (*neo*) and for the mouse dihydrofolate reductase gene (*DHFR*), each under control of the SV40 early promoter (Choo *et al.*, 1986). The *neo* marker confers resistance to the antibiotic G418 and the amplifiable *DHFR* marker allows selection for methotrexate (MTX) resistance. Genomic versions of the mouse *mdm1* (22 kb), *mdm2* (24 kb), and *mdm3* (36 kb) sequences were cloned into this vector for use in DNA transfection protocols. Each transfected gene included all coding exons and transcription termination signals, with expression driven by its own promoter. The source of the genomic DNA fragments was one of two cosmid libraries of 3T3DM DNA constructed either in the vector pCV001 or in the vector pWE15 (Wahl *et al.*, 1987). To construct these libraries, 3T3DM DNA was subjected to partial *Mbo*I digestion and fragments in the size range 25–50 kb were extracted from agarose gels and ligated into the *Bam*HI site of the vector. Recombinant clones were introduced into *E.coli* strain DH5 for storage and propagation. From these libraries were isolated genomic clones representing *mdm1*, *mdm2*, and *mdm3*. The *mdm1* expression construct was obtained directly from the 3T3DM/pCV001 cosmid library. The *mdm2* and *mdm3* genomic DNAs

were initially isolated from the pWE15-derived library and then transferred into the pCV001 vector at either the *Not*I site (*mdm3*) or the *Bam*HI site (*mdm2*).

Two independent 3T3DM cDNA libraries were constructed from poly(A)$^+$ RNA in the vector λZAP (Stratagene) according to published protocols (Gubler and Hoffman, 1983). Screening of genomic and cDNA libraries for *mdm2* clones initially used a probe derived from *mdm2* clone pdm66 (Cahilly-Snyder *et al.*, 1987). Additional rounds of screening were carried out to extend the overlapping cDNA sequences in the 5′ and 3′ directions.

DNA transfections and amplification

Cosmid DNA (20 μg) was transfected into half-confluent plates (100 mm) of NIH3T3 or Rat2 cells by the calcium phosphate coprecipitation protocol (Graham and Van der Eb, 1973). Approximately 48 h after transfection, the cells were passaged at a ratio of 1:4 into selective medium containing G418 (Gibco) at 400 μg/ml. Surviving transfected clones were pooled and subjected to step-wise selection (two-fold increases) in MTX from an initial MTX concentration of 0.05 μM to a final concentration of 0.4–0.8 μM as previously outlined (Choo *et al.*, 1986). Efforts were made to keep the cells subconfluent to minimize enrichment for spontaneously transformed cells.

Nucleotide sequencing

Genomic and cDNA clones were sequenced by the dideoxy method (Sanger *et al.*, 1977) using protocols for double- or single-stranded DNA fragments in plasmid cloning vectors pBluescript SK (Stratagene) or pGEM3 and pGEM4 (Promega). For some DNA fragments, sequencing was carried out on sets of nested deletions that were derived using an exonuclease III/mung bean nuclease system (Promega).

Tumorigenicity assays

For assays of tumorigenicity, 1×10^6 or 5×10^6 cells suspended in 0.2 ml serum-free MEM were injected subcutaneously into 4–6 week-old female NCR athymic (nude) mice. Animals were monitored regularly for tumor occurrence and size.

RNA isolation, Northern blots and Southern blots

Total RNA was isolated from cultured cells with guanidine hydrochloride as described (Liu *et al.*, 1979) and poly(A)$^+$ RNA prepared by chromatography through oligo(dT) cellulose. RNA from tissue samples was prepared using RNAzol, according to the recommendations of the supplier (Cinna Biotex). Northern blots and Southern blots were prepared following previously published protocols (George and Powers, 1981; George *et al.*, 1985), modified slightly to include the prehybridization of nitrocellulose filters in the presence of heparin (Singh and Jones, 1984). RNA filters were washed in 0.3 × SSC, 0.1% SDS at 55–60°C. (1 × SSC is 0.15 NaCl, 0.015 M sodium citrate.) DNA blots were washed under stringent conditions (0.1–0.2 × SSC, 60–65°C).

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