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Dissection of the Mouse N-*ras* Gene Upstream Regulatory Sequences and Identification of the Promoter and a Negative Regulatory Element

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The 5′ flanking region of the mouse N-*ras* gene was investigated to determine the elements governing transcriptional activity of the gene. The promoter did not contain typical TATA or CCAAT boxes, and according to primer extension and RNase protection analyses, transcription started at several sites. These assays also confirmed the short nucleotide distance interposed between the N-*ras* transcription unit and the previously described upstream *unr* gene. Chromatin studies performed by digestion of nuclei with DNase I revealed the presence of four hypersensitive sites: a, b, c, and d. Deletion mutagenesis of the 5′ flanking region revealed sequences responsible for both promotion and inhibition of transcription. These sequences resided within 230 bp upstream of the transcription initiation site. Hypersensitive site b colocalized with the 76-bp segment with promoter activity. The negative regulatory element at position −180 colocalized with hypersensitive site a, was active on the N-*ras* promoter in stable as well as transient assays, and down-regulated the heterologous herpes simplex virus thymidine kinase promoter. Footprint analysis and in vivo transfection-competition experiments indicated that a *trans*-acting factor is responsible for the negative effect on transcription. The interaction between the *cis*-acting negative regulatory element and the promoter region may play a role in the tissue- and developmental-stage-specific patterns of expression of the N-*ras* gene.

ras genes are members of a multigene family that is conserved from yeasts to humans at both the nucleic acid and amino acid levels (2), suggesting essential cellular functions for their products. Proteins coded for by these genes localize to the inner aspect of the plasma membrane and are able to bind, exchange, and hydrolyze guanine nucleotides (2). Although their specific function is still unknown, they can induce proliferation (13, 38) and influence cellular differentiation (3, 19, 39). In mammals, three functional *ras* genes (N-*ras*, c-K-*ras*2, and c-H-*ras*1) have been extensively characterized. They code for very similar, but not identical, proteins, with the majority of the amino acid changes occurring at the carboxyl end (2). Differences have also been observed at the level of RNA expression in different adult mouse tissues and during pre- and postnatal development (31). These observations are consistent with the hypothesis that the proteins coded for by the individual *ras* genes have different functions.

Alterations of *ras* gene expression are important for human tumorigenesis. *ras* genes activated by point mutations have been found in approximately 20% of the human tumors examined (2). Moreover, increased expression of normal N-*ras* and H-*ras* p21 proteins can cause transformation of transfected NIH 3T3 (7, 34) and, moderately, of rat-1 (43) cells. These observations suggest that overproduction of N- and H-*ras* proteins contributes to tumorigenesis. Studies of N-*ras* gene expression in murine tissues reveal high levels of the specific RNA in testis and thymus, while low or undetectable amounts are found in kidney, salivary gland, liver, and brain (28, 31). During development, N-*ras* also undergoes significant changes in expression (31). These observations indicate that the N-*ras* gene must be carefully regulated to respond to different physiological needs and to avoid the risk of overexpression, which can lead to cellular transformation (41, 46).

In an effort to understand the mechanisms regulating expression of the mouse N-*ras* gene, structural and functional analyses of the 5′ flanking region were carried out. In this study, the promoter region and the sites for initiation of transcription are defined. Furthermore, an upstream region transcribed by the *unr* gene (28) had a negative effect on transcription directed from the N-*ras* promoter as well as from heterologous promoters.

MATERIALS AND METHODS

DNase I hypersensitivity. Nuclei from NIH 3T3 fibroblasts and from a C57BL/10 thymic lymphoma cell line were prepared as described elsewhere (10) and assayed for sites sensitive to digestion by DNase I according to the end-labeling technique (51) with some modifications. Concentrations of DNase I used were from 0.5 to 4.0 U (1 U = 0.57 μg) per 0.5-ml aliquot of sample. Digestions of extracted nuclei were done at room temperature for 3 min and stopped by the addition of 0.05 ml of 5% sodium dodecyl sulfate–100 mM EDTA. Southern blot analysis was performed as described previously (48). Probes were labeled with [α-^{32}P]dCTP by the random primer method. Filters were hybridized at 42°C and washed at 60°C in 0.4× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate [pH 7.2]).

Oligonucleotides. The following oligonucleotides were used for double-stranded DNA in gel retardation and in vivo competition experiments: wt22 (5′-CCGGTTTTATGTT AATGGCGAA-3′), 5′d-20 (5′-TCCGGT*GTTAATGGCGA AAG-3′), and 3′d-20 (5′-TCCGGTTTTATGT*GCGAAAG-3′). wt22 is from the region −193 to −172. 5′d-20 and 3′d-20 are from the region −194 to −170 and contain an internal

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deletion of 5 bases within the footprinted region. For single-stranded DNA for primer extension, the oligonucleotides were a (5′-CGGGAGGCTTTTGGACTTCA-3′), which is the inverted complement of the sequence between nucleotides +73 and +93, and b (5′-AACCACCACCAGTTTGTACTC AGTCAT-3′), which is the inverted complement of the sequence between +588 and +615 of exon I.

Electrophoretic mobility shift assay. DNA-binding and electrophoretic mobility shift analyses were performed as described elsewhere (6). NIH 3T3 and HeLa nuclear extracts were prepared by the method of Dignam et al. (11) and concentrated by precipitation with ammonium sulfate. One microliter of end-labeled DNA fragments (specific activity, 5×10^8 cpm/μg) was mixed with 20 μg of poly(dI-IC):poly(dI-dC) (Pharmacia, Piscataway, N.J.) in a volume of 16 μl. Nuclear extracts (20 μg) were then added in a volume of 20 μl of buffer D (11) containing 10 μg each of pepstatin and aprotinin per ml, and binding was allowed to proceed at 4°C for 20 min. DNA-protein complexes were resolved by electrophoresis at 4°C on prerun 4% polyacrylamide gels at 30 mA for 2 h.

DNA footprinting. Binding reactions and footprinting analysis were performed as described elsewhere (6). DNA-protein-binding reaction mixtures were treated with 1 μl of DNase I (50 μg/ml) at 4°C for 60 s. Reactions were stopped by the addition of 10 mM EDTA, and the mixtures were immediately loaded on a prerun 4% polyacrylamide preparative gel. Protein-DNA complexes were localized by autoradiography of the wet gels at 4°C. Bands corresponding to free and complexed probes were excised, DNA electroeluted, and ethanol precipitated. Samples were analyzed on 8% polyacrylamide–7 M urea sequencing gels.

RNA extraction, RNase protection, and primer extension analysis. Total RNA was isolated from cell lines and tissues by the guanidium thiocyanate-cesium chloride method (9). Uniformly ^{32}P-labeled RNA probes were synthesized with SP6 polymerase from cloned N-*ras* fragments inserted into pGEM vectors (Promega, Madison, Wis.). RNase protection experiments were performed by the method of Melton et al. (35), with the following modifications. Total cellular RNA (30 μg) was hybridized to labeled probes at temperatures of 50 to 55°C for 16 h and then digested with RNase A and RNase T1 at 30 to 37°C. Primer extension was performed by end labeling the primers with [γ-^{32}P]ATP and annealing them to 50 μg of total RNA at 25°C, according to established protocols (44). Moloney murine leukemia virus was used as reverse transcriptase. Samples were analyzed on 6% denaturing acrylamide gels.

Northern (RNA) blot analysis. Total RNA (12 μg per lane) was electrophoresed on 1% agarose-formaldehyde gels and transferred to nitrocellulose filters according to standard procedures (44). Filters were hybridized at 42°C and washed at 60°C in 0.4× SSC.

Plasmid constructions. The transforming mouse N-*ras* gene (8 kb), mutated at codon 61 (18), was subcloned into the *Xho*I site of Bluescript (Stratagene, La Jolla, Calif.) and designated pNT8kb. Deletions of the 5′ flanking region were generated by digestion with exonuclease III followed by S1 nuclease treatment (22). All deletion endpoints were determined by sequencing. Plasmid p110, containing 1.1 kb of 5′ flanking region linked to transforming N-*ras*, was constructed as follows. pNT8kb was partially digested with *Bal*I to yield a 6.5-kb *Bal*I-*Xho*I fragment including the last three coding exons of the gene. The 6.5-kb fragment was ligated to *Xho*I linkers and subcloned into Bluescript (pNT6.5kb). A 2.5-kb *Bal*I-*Bal*I fragment containing 1.1 kb of the 5′ flanking region and the first exon of the gene was derived from *Bal*I digestion of a pBR plasmid containing the normal N-*ras* gene (18). This 2.5-kb *Bal*I-*Bal*I fragment was then subcloned into pNT6.5kb by means of *Xho*I linkers to yield p110. Plasmid pNRE-tkCATu was constructed by subcloning the *Hae*III-*Mae*I fragment from the 5′ flanking region of N-*ras* into the *Bam*HI site of pBLCAT2 (33). p215-tkCAT generated with a 215-bp fragment from the 3′ end of the K-*fgf* gene was obtained from A. Curatola. This fragment has been shown not to have an effect on transcription (10a).

Transfection, focus formation assay, and G418 assay. NIH 3T3 and HeLa cells were grown in monolayer cultures at 37°C in an atmosphere of 5% CO_2 and maintained in Dulbecco's modification of Eagle's medium supplemented with 10% bovine serum (GIBCO, Grand Island, N.Y.). The transforming activity of the N-*ras* deletion mutants was determined by the focus formation assay on NIH 3T3 fibroblasts (17). Plasmid pNEO3, containing the neomycin resistance gene (4), was used as an internal control. One day before transfection, cells were seeded at 3×10^5 cells per 100-mm dish. Calcium phosphate DNA precipitates containing 50 ng of the test plasmid, 50 ng of pNEO3, and 20 μg of carrier DNA was added to each plate. Fresh medium was added after 16 h, and cells were further incubated for 10 h. At 26 to 28 h after the addition of DNA, each dish was trypsinized and replated into nine 100-mm dishes, of which six were fed with medium containing 5% bovine serum for selection of foci. The remaining three dishes were fed with medium containing 10% bovine serum and 400 μg of G418 per ml. After 2 weeks, dishes were fixed and stained for scoring of either foci or G418-resistant colonies.

Transfections for transient expression were performed on HeLa cells with 5 μg of test plasmid. To normalize for transfection efficiencies, plasmid pCH110 (21) or plasmid pSV2CAT (15) was cotransfected. Carrier DNA was added to a total of 20 μg per transfection. Cells were harvested 48 h after the addition of DNAs for either RNA or protein extractions.

CAT assay. Cells were washed with phosphate-buffered saline, scraped, and centrifuged. Pellets were suspended in 100 μl of 0.25 M Tris HCl (pH 7.5), subjected to four cycles of freeze-thaw, and centrifuged at 10,000 × *g* for 10 min. Supernatants were normalized according to their β-galactosidase activity (23), and chloramphenicol acetyltransferase (CAT) activity was determined as described elsewhere (15).

RESULTS

5′ Region of mouse N-*ras* gene contains sites hypersensitive to DNase I digestion. Sites of chromosomal DNA sensitive to digestion by DNase I are frequently associated with regions that are functionally important for the expression of transcriptionally active genes (16). Such sites include, among others, enhancer, silencers, and promoter regions. As a first step toward determining the elements involved in transcriptional regulation of the mouse N-*ras* gene, the chromatin structure of the gene was analyzed for sensitivity to DNase I. As shown in Fig. 1, digestion of purified nuclei with increasing concentrations of the enzyme followed by probing with a 350-bp fragment (probe A from the 3′ end of the restriction fragment *Eco*RI-*Sac*I) revealed the presence of four DNase I-hypersensitive sites at the 5′ end of the gene. The most distal site (HS-a), corresponding to the 1.1-kb band, was located approximately 150 bp upstream of the 5′ untranslated exon. A second DNase I-hypersensitive site (HS-b) partially overlapped the same untranslated exon and

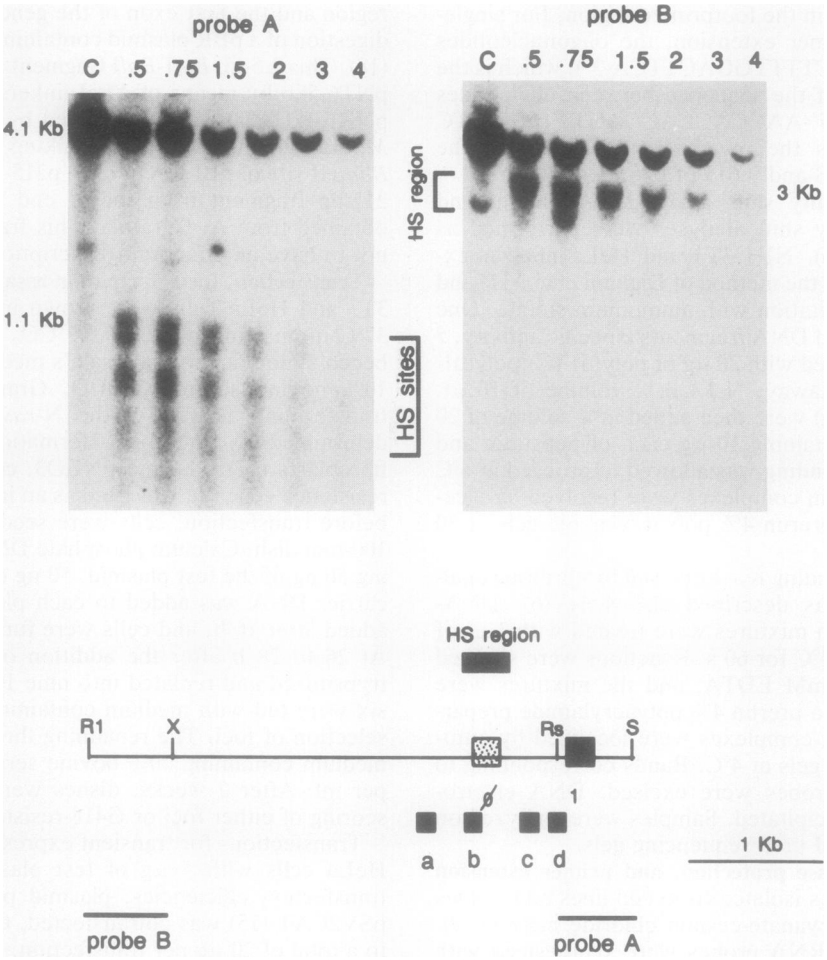

FIG. 1. Localization of chromosomal sites hypersensitive to digestion with DNase I. A thymic lymphoma cell line from C57BL/10 mice was used. Southern blot was done with 30 μg of purified DNA per lane. The DNA, derived from DNase I-treated nuclei, was digested with *Sac*I-*Eco*RI. Left panel, Hybridization to probe A; right panel, the same filter as in the left panel was stripped and rehybridized to fragment *Xba*I-*Eco*RI (probe B). Symbols and abbreviations: ■, translated exons; ▨, untranslated exon; Ø, 5′ untranslated exon (Phi) of N-*ras*; S, *Sac*I; R1, *Eco*RI; X, *Xba*I; Rs, *Rsa*I. The positions of HS-a, -b, -c, and -d are indicated by smaller filled boxes.

extended approximately 50 bp further upstream. Two other hypersensitive sites (HS-c and HS-d) were located within the first intron of the N-*ras* gene. A similar pattern of DNase I hypersensitivity was observed with nuclei from NIH 3T3 fibroblasts (not shown).

The N-*ras* HS sites correlate with formation of specific DNA-protein complexes in vitro when DNA probes containing HS-a, HS-b, HS-c, and HS-d and extracts from NIH 3T3 cells were used in DNA mobility shift assays (not shown). Therefore, the pattern of hypersensitivity of the promoter region is conserved in different cell types and correlates with formation in vitro of specific DNA-protein complexes, indicating that these regions could be important for the regulation of the N-*ras* gene.

Multiple sites for initiation of transcription are identified for the N-*ras* gene. By using RNase protection experiments and three different probes we detected multiple sites for transcription initiation (Fig. 2). Probe 442, which spans the 5′ end of the N-*ras* gene and the 3′-most region upstream of the N-*ras* (*unr*) gene (28), yielded a cluster of intense protected bands 214 to 225 nucleotides (nt) in size as well as smaller bands ranging from 70 to 100 nt (Fig. 2). Probes spanning smaller segments within this region were used to identify the origins of the various protected bands. Probe 151 (3′-most end of probe 442), which partially overlapped the 5′ untranslated exon of N-*ras*, yielded only the smaller (70- to 100-nt) protected bands and identified the sites for initiation of transcription, while probe 291 (5′-most end of probe 442) yielded only the larger (214- to 225-nt) protected bands and identified the products of the *unr* gene.

Primer extension analysis was performed to confirm these results. Two independent primers were used: a from exon I and b from the 5′ untranslated exon Phi. Products 155 nt long with primer a and 90 nt long with primer b were expected if the position +1 identified by RNase mapping (the 88- to 89-nt bands obtained with probes 442 and 151) was correct. The results obtained correspond quite closely with these predictions (Fig. 2B).

In addition to the 155-nt bands, primer a yielded smaller products. The 140-nt band corresponds to an initiation site at +16 (Fig. 3) and was also found with probe 442 and 151 in RNase protection experiments (the 72- to 74-nt bands). This position corresponded to a major site of initiation when probe 151 and RNA from tissues (testis and thymus) were

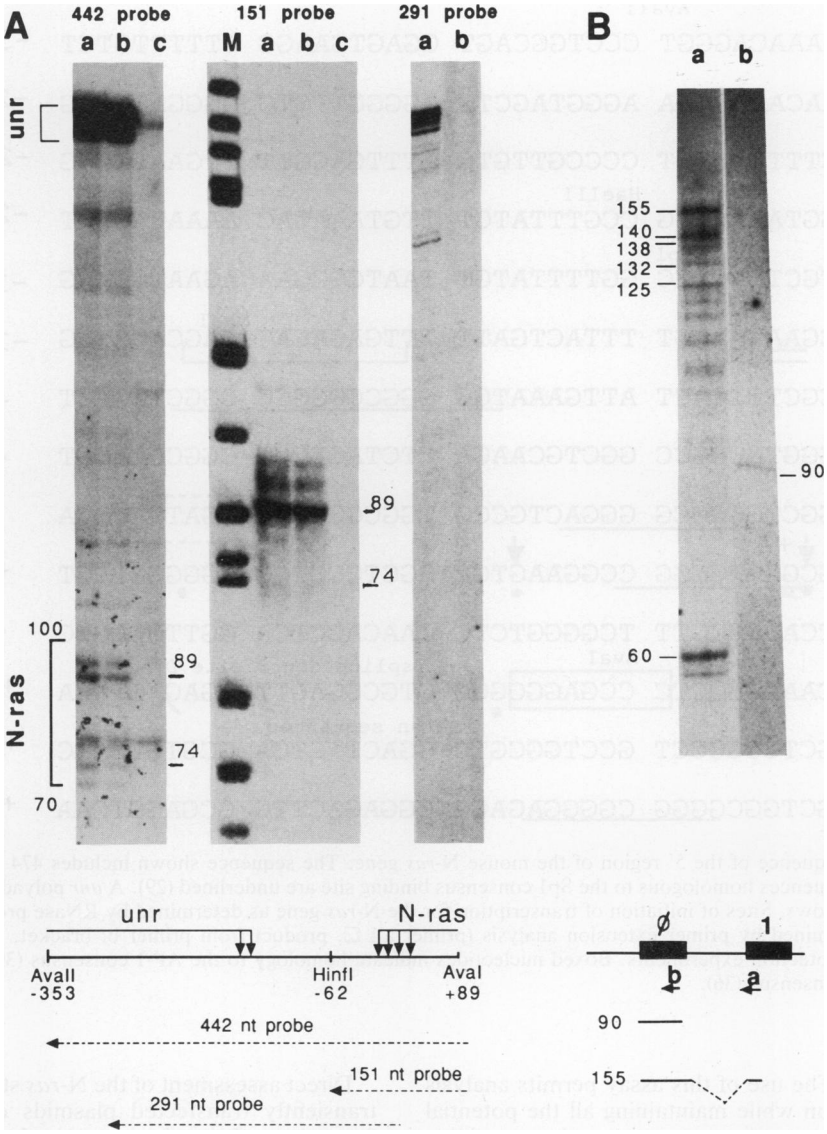

FIG. 2. Determination of N-*ras* transcription initiation. (A) RNase protection analysis performed as described in Materials and Methods with the three probes indicated. Probe 442 is an antisense RNA probe overlapping the downstream region of the *unr* gene and the upstream region of the N-*ras* gene. RNA from NIH 3T3 fibroblasts was hybridized to the corresponding probes and digested with RNase A and RNase T1, and fragments were analyzed on a sequencing gel. Probe 151 corresponds to the 3′-most 151 nucleotides of probe 442 and overlaps only the N-*ras* gene. For probes 442 and 151: lane a, RNase digestion at 30°C; lane b, RNase digestion at 37°C; lane c, yeast tRNA; lane M, marker (end-labeled fragments of pBR322 digested with *Hae*III). Probe 291 corresponds to the 5′-most 291 nucleotides of probe 442 and covers only the *unr* gene. For probe 291: Lane a, RNase digestion at 30°C; lane b, yeast tRNA. (B) Lane a, Primer extension analysis performed with primer a from exon I; lane b, products from primer b. Total RNA (50 μg) from NIH 3T3-3.2 cells expressing high levels of N-*ras* transcripts (18) was used. To determine the molecular weights of the products, all reactions were run next to a sequencing reaction.

examined (not shown). Position +97, corresponding to the 60-nt band, represents a new strong site not revealed by RNase protection experiments because it is located downstream of probes 151 and 442. Other smaller bands of lower intensity observed with primer a do not have corresponding bands in RNase protection experiments and could represent stop sites for reverse transcriptase rather than additional transcription initiation sites.

Primer b from exon Phi additionally confirms the start site at position +1 (the 90-nt band). The absence of lower-molecular-weight bands with this primer could be caused by nonspecific hybridization of the primer, which subtracts intensity from the specific signal; therefore, only the most abundant RNA species would be detected.

Taken together, these results show that N-*ras* transcription starts at multiple sites. The stronger upstream site is referred to as nucleotide +1.

Promoter and negative regulatory element (NRE) control expression of the gene. To functionally define the promoter region of the N-*ras* gene, a series of deletion mutants which lacked progressively larger fragments of the 5′ flanking region was constructed. The importance of these regions for expression of the gene was tested by using the transforming N-*ras*$^{Lys-61}$ as a reporter gene in a focus formation assay on

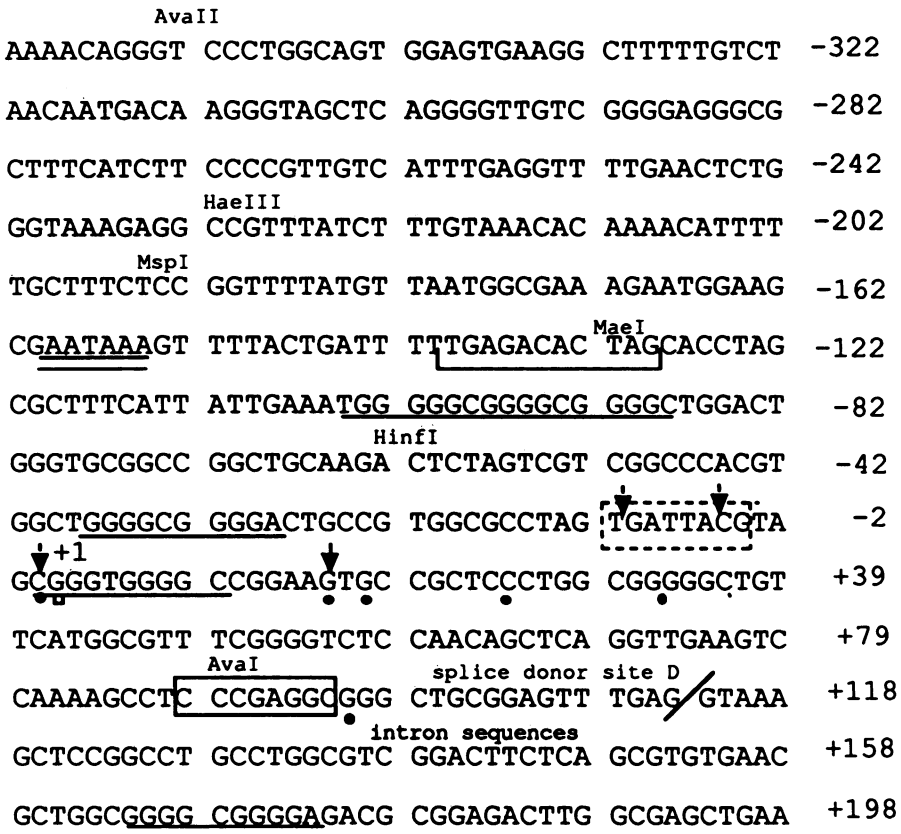

FIG. 3. Nucleotide sequence of the 5′ region of the mouse N-*ras* gene. The sequence shown includes 474 nucleotides upstream of the splice donor site (D). Sequences homologous to the Sp1 consensus binding site are underlined (29). A *unr* polyadenylation signal (AATAAA) is double underlined. Arrows, Sites of initiation of transcription for the N-*ras* gene as determined by RNase protection; ●, site of initiation of transcription as determined by primer extension analysis (primer a); □, product from primer b; bracket, 3′ end of *unr* transcripts as determined by RNase protection experiments. Boxed nucleotides indicate homology to the AP-1 consensus (30); the dashed box indicates homology to the AP-2 consensus (36).

NIH 3T3 fibroblasts. The use of this assay permits analysis of the 5′ flanking region while maintaining all the potential regulatory elements internal to the gene, thus avoiding complexities arising from the addition or removal of elements that could result from the use of other reporter genes.

Deletion mutants containing 76 bp of 5′ flanking sequences (plasmid p329) retained transforming efficiencies equivalent to those of control wild-type constructs (Fig. 4A). A more extensive deletion, up to nucleotide −19, in plasmid p27 eliminated a sequence with high homology to the Sp1-binding site (29) and decreased the activity of the plasmid by 70%, indicating that sequences important for efficient transcription were deleted. Loss of sequences upstream of position +30 (plasmid p6B) abolished virtually all transforming activity. Thus, a region located 76 bp upstream of the N-*ras* cap site harbors the promoter sequences for the gene that are essential for efficient transcriptional activity. This region overlaps DNase I-hypersensitive site HS-b defined above.

Deletion mutant p5A, which contained, in addition to the 76-bp promoter region, upstream sequences extending up to position −221, showed a decreased efficiency in focus formation assays. Repeated transfections with independent preparations of plasmid p5A yielded the same results, arguing against the presence of contaminants from the plasmid purification process.

Direct assessment of the N-*ras* steady-state RNA levels of transiently transfected plasmids demonstrated that focus formation efficiencies correlated well with transcriptional activity of the deletion mutants (Fig. 4B). Construct p5A reproducibly yielded decreased (45% of wild-type) levels of N-*ras* transcripts. The region from position −221 to −153 present in plasmid p5A corresponded to the location of HS-a previously determined. Taken together, these observations suggest the presence of a *cis*-acting element, located 221 and 153 bp upstream of the N-*ras* cap site, that negatively modulates transcriptional activity of the gene both in NIH 3T3 fibroblasts and in HeLa cells. This region is contained in the transcribed sequences of the *unr* gene (Fig. 3).

N-*ras* NRE is active on heterologous promoters. To test whether the NRE from N-*ras* had reducer activity on heterologous promoters, constructs were generated which linked the 100-bp fragment containing the NRE to the promoter from the herpes simplex virus thymidine kinase gene (*tk*) in plasmid pBLCAT2. In pNRE-tkCATu constructs, the activity of the *tk* promoter was decreased to 43 to 59% of that of control constructs (without the 100-bp fragment or with unrelated DNA sequences) (Table 1). This effect was independent of the orientation of the element, since both sense and antisense NRE reduced CAT activity, but dependent on distance from the promoter. The reducing effect was additive, since three copies of the 100-bp fragment in p3NRE-

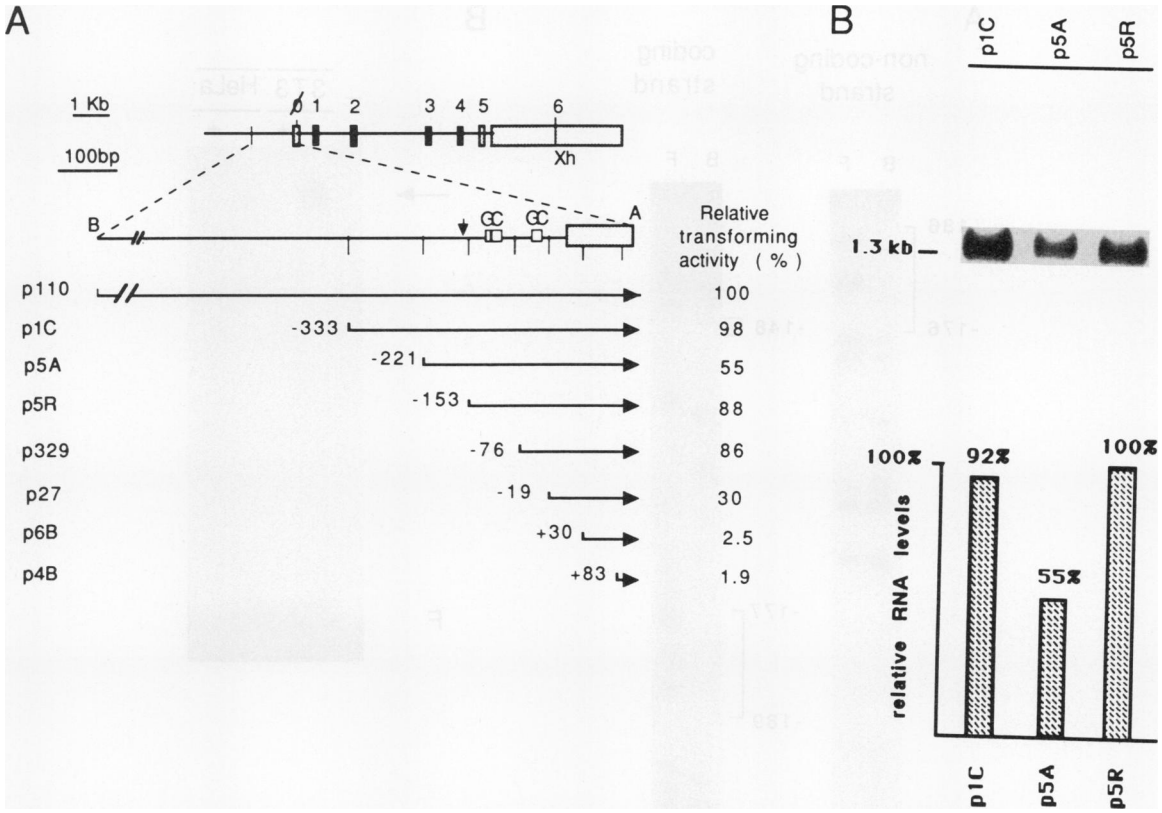

FIG. 4. Stable and transient transfections with deletion mutants of the N-*ras* gene. (A) The structure of the entire N-*ras* oncogene is shown at the top. Coding and noncoding exons are represented as in Fig. 1. Sp1 consensus repeats (GC boxes) in the flanking region are indicated. Arrow, Position of a polyadenylation signal for the *unr* gene. To the right, relative transforming activities are expressed as percentages of the mean obtained with plasmid p110. Numbers shown are averages of five independent transfection experiments. Plasmid p110 efficiency of transformation was 22 transformants per ng of DNA per 3×10^6 cells. Abbreviations: B, *Bal*I; A, *Ava*I; Xh, *Xho*I. (B) Top, Northern blot analysis of N-*ras* transcripts in cells transiently transfected with deletion mutants. Twelve micrograms of total RNA was used. The probe was an *Eco*RI fragment of pSUN (cDNA from the 3′ untranslated end) (31). Bottom, Laser scanning densitometry of autoradiograms from Northern blot shown at the top. Results were normalized relative to the signal given by CAT transcripts produced by cotransfected pSV2CAT plasmid and detected by reprobing the same filter used at the top for N-*ras* transcripts with a CAT probe. Values given are from a representative experiment. Repeated transfection experiments yielded equivalent results.

tkCAT reduced the *tk* promoter activity by 73%. No effect on efficiency of transcription was observed when the fragment was placed in front of the simian virus 40 enhancer-promoter (not shown), probably because of the potency of the simian virus 40 enhancer.

TABLE 1. Determination of transcriptional activity of the N-*ras* NRE on the thymidine kinase promoter

Reporter plasmid[a]	Orientation[b]	Relative CAT activity
pBLCAT-2		100
pNRE-tkCATu	A	43 ± 2.6
pNRE-tkCATu	S	59 ± 2.5
p3NRE-tkCAT		27 ± 2.3
pNRE-tkCATd	A	78 ± 6.3
pNRE-tkCATd	S	85 ± 2.8
p215-tkCAT		94 ± 14

[a] NRE fragment *Hae*III-*Mae*I was subcloned into the *Bam*HI site of plasmid pBLCAT2 (33) to give pNRE-tkCATu. pNRE-tkCATd contained the NRE downstream of the CAT gene.

[b] A, Antisense; S, sense.

[c] HeLa cells were transfected and harvested after 48 h, and CAT activity was determined. Samples were normalized for levels of β-galactosidase activity expressed from the cotransfected plasmid pCH110. Values represent averages of six independent transfection experiments.

Thus, the N-*ras* NRE is able to reduce not only transcriptional activity directed from its own promoter but also that directed from at least one heterologous promoter in an orientation-independent manner.

Specific DNA-protein complexes are localized within the N-*ras* NRE. Since the region containing the NRE is extraordinarily conserved in evolution (8, 20, 28) and since no sequences similar to that of the NRE were observed in the regions upstream of the H-*ras* or K-*ras* genes, this element could play a role in the specific regulation of N-*ras* gene expression. The presence of HS-a in vivo (Fig. 1) and the formation of DNA-protein complexes in vitro with the 100-bp *Hae*III-*Mae*I probe (not shown) indicated that a protein factor(s) was specifically interacting with NRE.

DNA footprint analysis (Fig. 5) revealed protected minimal regions of 12 nt in the coding strand and 10 nt in the noncoding strand. A synthetic double-stranded oligonucleotide (wt22), encompassing the sequences protected by footprint, efficiently competed with the 100-bp NRE probe in the formation of the shifted complex (not shown). When the wt22 oligonucleotide was used as probe in DNA mobility shift experiments (Fig. 5B), a specific complex was observed with extracts from NIH 3T3 and HeLa cells. These results

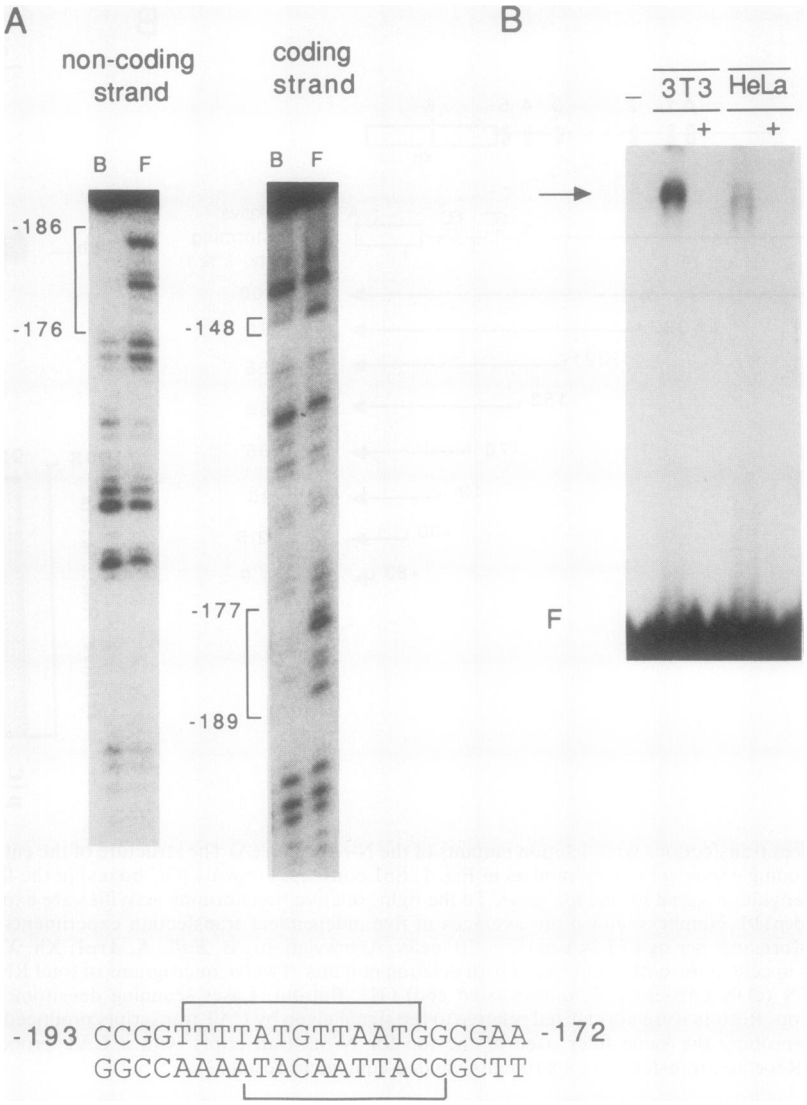

FIG. 5. Localization of specific binding activities in the NRE fragment. (A) Footprinting analysis of the bound complex formed with the NRE probe was performed as described in Materials and Methods. For the coding strand, a *Hae*III-*Hae*III fragment was end labeled and digested with *Mae*I to give the 100-bp *Hae*III-*Mae*I probe. DNA-protein reactions were electrophoresed in a preparative polyacrylamide gel. Radioactive fragments from the complex (lane B) and from the free-migrating band (lane F) were recovered as described in Materials and Methods. For the noncoding strand, the *Hae*III-*Mae*I fragment was end labeled and digested with *Msp*I. Numbers on the left indicate the distances in nucleotides of the protected regions from the N-*ras* major cap site. Bottom, Oligonucleotide wt22. (B) Oligonucleotide wt22 was used as probe in DNA mobility shift experiments together with 5 μg of NIH 3T3 or HeLa nuclear extracts. Specificity of the complex is indicated by the competition of the retarded band (arrow) when a 100-fold excess of the unlabeled wt22 was added (+). F, Free probes.

indicate that the sequence between −189 and −176 in the N-*ras* promoter contains the NRE-binding site.

N-*ras* NRE repressor activity is inhibited by a 22-bp oligonucleotide. To investigate whether the protein(s) binding to NRE in a specific fashion is responsible for the negative effect observed on promoter transcription, we performed in vivo transfection-competition experiments. Cotransfection into HeLa cells of plasmid pNRE-tkCATu with the wt22 oligonucleotide that competed in vitro with the formation of the shifted complex yielded correspondingly increased levels of CAT activity (Table 2). In addition, when double-stranded oligonucleotides containing internal deletions of the NRE-binding site were used, no increase in CAT activity of the reporter plasmid was observed. As a control, cotransfection with equivalent molar amounts of a nonspecific DNA fragment had no effect on CAT activity. We conclude that a cellular factor(s) recognizing sequences from −189 to −176 in the N-*ras* promoter region is responsible for reducing the activity of promoters to which such sequences are linked in *cis*. The precise function of the N-*ras* NRE region remains to be investigated.

DISCUSSION

The structural and functional analyses of the 5′ region of the mouse N-*ras* gene carried out in this study have yielded information on sequences functionally relevant for the control of expression of the gene. Chromatin analysis coupled to

TABLE 2. In vivo competition analysis by cotransfection[a]

Reporter plasmid	Competitor DNA	CAT activity
pNRE-tkCATu(a)		1
pNRE-tkCATu(a)	wt22	2.4
pNRE-tkCATu(a)	5'd-20	1.2
pNRE-tkCATu(a)	3'd-20	0.9
pNRE-tkCATu(a)	122-bp *Ava-Hae*	1.3
pBLCAT2		2.3

[a] pNRE-tkCATu(a) (2.5 μg) was cotransfected into HeLa cells with 50-fold molar excess of the wt22 oligonucleotide together with 1 μg of pCH110 to normalize for transfection efficiencies. Other DNA competitors used are indicated.

deletion mutagenesis of the 5′ flanking region has identified the promoter of the gene together with a negative *cis*-acting element that specifically interacts with a *trans*-acting factor(s).

Structurally, the promoter resembles the H- and K-*ras* promoters: no TATA or CCAAT boxes are found at significant positions, while several copies of the Sp1-binding site together with a 5′ untranslated exon are present (20, 25–27, 52). In addition, the N-*ras* gene contains sequences homologous to the AP-1 (30) and AP-2 (36) consensus binding sites at positions −11 and +89, respectively, suggesting a possible specific regulation of the gene by these transcription factors.

Transcription starts at multiple sites, as indicated by both RNase mapping and primer extension analysis (Fig. 2). The farther-upstream major site, indicated as nucleotide +1, appeared more intense in NIH 3T3 (Fig. 2) and C57BL/10 thymic lymphoma cells (28), while site +16 was prevalently used in thymus and testis. The sites for transcription initiation found here correspond quite closely to those determined in hamsters (12). Probes 442 and 291 in the RNase protection assay also detected transcripts derived from the upstream *unr* gene (Fig. 2; 28), thus determining that only 117 nt divide the two transcription units. Presence of the *unr* gene, only recently acknowledged (12, 28), might have misled previous attempts to define the 5′ border of the human N-*ras* gene (20).

The functional assessment of sequences in the 5′ flanking region that are important for expression of the gene was obtained by focus formation assay. This assay has the advantage of taking into consideration potential intragenic regulatory elements that would have been missed by using chimeric constructs containing only the 5′ sequences to be tested linked to heterologous reporter genes. Such a situation has been encountered, for example, in studying the H-*ras* promoter (26, 27). Good correlation between N-*ras* transcript levels in transient assays and scoring in the focus formation assay (Fig. 5) supports the validity of stable transfection assays for analyzing transcriptional activity in our system (Fig. 4).

This analysis indicated that the promoter sequences located within 76 bp upstream of exon Phi can account for 86% of the transforming activity of the gene. Deletion of sequences between −76 and −19 in plasmid p27, which contains a conserved Sp1-binding site, decreased activity of the promoter by 70%, while presence of the farthest-upstream Sp1-binding sites in plasmid p5R did not further increase the activity of the promoter (Fig. 4). This situation is reminiscent of that found by Ishii et al. (27) in the H-*ras* gene, where only the proximal GC boxes were important for efficient promoter activity.

A negative *cis*-acting element (NRE) is also found at position −189 to −176. Reducer or silencer elements have been described in various mammalian genes (1, 5, 24, 37, 42, 45, 50). In most cases, such elements are also active on heterologous promoters. The N-*ras* NRE exerted a negative effect on transcription when it was placed 5′ of either the homologous promoter in stable or transient assays or the herpes simplex virus thymidine kinase promoter in transient assays (Fig. 4 and Table 1).

DNA-protein-binding assays and footprint analysis revealed that a *trans*-acting factor(s) binds to the NRE sequence (Fig. 5). In vivo transfection-competition experiments indicated that the *trans*-acting factor(s) binding to the NRE is responsible for the negative effect on promoter activity (Table 2). Oligonucleotides 5′d-20 and 3′d-20, which bear deletions of the sequences TTTAT (−188 to −184) and TAATG (−181 to −177), respectively, did not form a specific DNA-protein complex in retardation assays (not shown), nor were they able to relieve the down-regulation of the *tk* promoter when cotransfected with the pNRE-tkCATu reporter plasmid (Table 2). These results indicate that sequences between −188 and −177 are important for NRE activity. On the other hand, when nucleotides −184 to −185 were point mutated and the resulting 100-bp NRE altered regions were tested upstream of *tk* promoter-CAT, the activity of these plasmids was very similar to that of pNRE-tkCATu containing the wild-type NRE (39a), indicating that those nucleotides were not important for NRE activity.

Eukaryotic transcriptional repressor factors represent a growing category of regulatory proteins (32). The N-*ras* NRE (5′-GTTTTATGTTAATGG-3′) shows homology with the negative regulator of the albumin enhancer ANF A (5′-GTTTTCTTTATCTGG-3′) (24) and with the octa sequence in the chicken myosin heavy-chain promoter (5′-TATGCAAAT-3′), which also functions in down-regulating transcription (49). The NRE sequence is highly conserved among human, rat, and mouse genes (97 to 99%) (8, 20, 28). These observations suggest the importance of the N-*ras* NRE in regulation of the endogenous gene. In particular, the NRE could be associated with the specific down-regulation of the gene seen in certain tissues or the sharp decrease in RNA levels observed around day 16 of mouse fetal development (28, 31). Moreover, NRE activity might be critical during germ cell development, when N-*ras* remains silent until the meiotic phase, while in the postmeiotic phase, a specific switch occurs from K-*ras* to N-*ras*, possibly indicating a need for a different target specificity during gametogenesis (40, 47). Mutational manipulation of the element and analysis of "inappropriate" expression of the N-*ras* gene in transgenic mice are currently two of our approaches to answering the questions about the specific roles for NRE in vivo. It is conceivable that N-*ras* gene regulation is a complex process like eukaryotic gene regulation, in which multiple positive and negative elements overlap (14) or act in concert (1, 5, 24, 45), and that all sequences and factors must be elucidated before we can fully understand complex regulatory circuits like the N-*ras* gene.

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