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A Short, Highly Repetitive Element in Intron −1 of the Human c-Ha-*ras* Gene Acts as a Block to Transcriptional Readthrough by a Viral Promoter

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We have identified a short, highly repetitive element within intron −1 of the human c-Ha-*ras* gene. This element was found to be transcribed in both orientations and to be homologous to heterogeneous nonpolyadenylated transcripts. The repetitive element blocked transcriptional readthrough from a strong upstream viral promoter but allowed unimpaired readthrough from the c-Has-*ras* promoter. We suggest that it may serve to prevent excessive transcription into the coding region of the gene under such circumstances as viral insertion.

Elevated expression of the c-Ha-*ras* gene is associated with many important tumor types, such as breast (28), colon (13), and gastric (4). In addition, elevated expression of the normal gene under the control of a retrovirus long terminal repeat has been shown to induce tumorigenic transformation of 3T3 cells (7), and activation of the c-Ha-*ras* oncogene in an avian nephroblastoma has been attributed to proviral insertion (30).

The potential importance of c-Ha-*ras* transcriptional deregulation in tumor progression led us to examine the nature of the c-Ha-*ras* promoter and the factors which regulate its expression (20), and we have recently identified a bidirectional c-Ha-*ras* promoter whose 3′ end (the donor splice site) is located 1,105 base pairs (bp) upstream of the ATG codon. The direct promoter contains 18 initiation sites between −196 and −16 bp relative to the donor splice site (D). The reverse promoter contains nine initiation sites between −248 and −278 bp relative to D. A 43-bp element between −243 and −196 up regulates activity of the direct promoter by 20- to 40-fold (20; summarized in Fig. 1c).

A highly repetitive, transcribed element in c-Ha-*ras* intron −1. To confirm that the far upstream exon −1 promoter gives rise to mature 1,400-bp c-Ha-*ras* mRNA, we performed Northern (RNA) blot analysis, using probes derived from exon −1 and the coding exon 1. Total RNA was prepared from EJ bladder carcinoma cells (23) and the human erythroleukemia cell line K562 (26). Probes derived both from exon −1 and exon 1 detected a major 1,400-bp c-Ha-*ras* mRNA species and a minor species of approximately 5.5 kilobase pairs (kb) (Fig. 1a). To determine whether the larger RNA species represented a splicing intermediary, a 1.16-kb *Sau*3A fragment containing intron −1 was used as a hybridization probe. Surprisingly, this probe hybridized to highly repetitive heterogeneous RNA species ranging in size from approximately 7 kb to 800 bp. We proceeded to localize the DNA sequence coding for repetitive RNA by using a series of probes derived from intron −1. The 837-bp *Xma*III-*Sau*3A probe (containing the 3′ end of intron −1 and coding exon 1) hybridizes to c-Ha-*ras* 1,400-bp mRNA, whereas the 305-bp *Xma*III probe detects the repetitive species. A 130-bp *Xma*III-*Sst*II fragment from the extreme 5′ end of intron −1 also hybridizes to repetitive RNA, whereas the 170-bp *Sst*II-*Xma*III probe does not. The repetitive RNA species are mainly represented in the nonpolyadenylated RNA fraction. Using multiple overlapping probes, we have confirmed that the entire length of the 130-bp *Xma*III-*Sst*II fragment hybridizes to repetitive RNA and that sequences immediately 5′ and 3′ of this fragment do not (not shown).

To determine whether the highly repetitive RNA species is derived from a single (or low-copy-number) DNA sequence or from highly repetitive DNA, 10 μg of genomic DNA from the EJ bladder carcinoma cell line was digested to completion with *Bam*HI or *Eco*RI, electrophoresed through 1% agarose, and transferred to nitrocellulose. Using an 837-bp *Xma*III-*Sau*3A probe and a 170-bp *Sst*II-*Xma*III probe, we detected a single-copy 6.4-kb *Bam*HI fragment and a single-copy 23-kb *Eco*RI fragment spanning the c-Ha-*ras* gene. A 305-bp *Xma*III probe and an 1,146-bp *Sau*3A probe hybridized to highly repetitive DNA ranging in size from 35 kb to 400 bp (Fig. 1b). By serial dilution of genomic DNA samples, we calculate that this element is repeated approximately 300,000 times in the genome. Computer analysis of this sequence reveals no resemblance to any known repetitive element.

Pattern of transcription within the intron −1 repetitive element. To analyze the transcription pattern from this highly repetitive element, we cloned a 460-bp *Sau*3A-*Sac*I fragment derived from intron −1 (in both orientations) into the *Bam*HI site of the promoterless chloramphenicol acetyltransferase (CAT) vector pCO (20) (Fig. 2a). Increasing amounts (from 0.1 to 20 μg of DNA per 75-cm^2 flask) of constructs 460D-CAT, 460R-CAT, and pCO were introduced by calcium phosphate precipitation (31) into Cos 7 cells; 48 h later, cell extracts were analyzed for CAT activity (15). The construct IE-CAT described by Lowndes et al. (20) (in which the herpesvirus immediate-early [IE] promoter is cloned into the *Bam*HI site of pCO) was included for comparison. Maximum CAT activity was generated by 460D-CAT and 460R-CAT at an input concentration of 10 μg per flask, whereas IE-CAT reached maximum activity at 0.5 μg per flask. CAT activity generated by the repetitive intron promoter was identical in both orientations and was approx-

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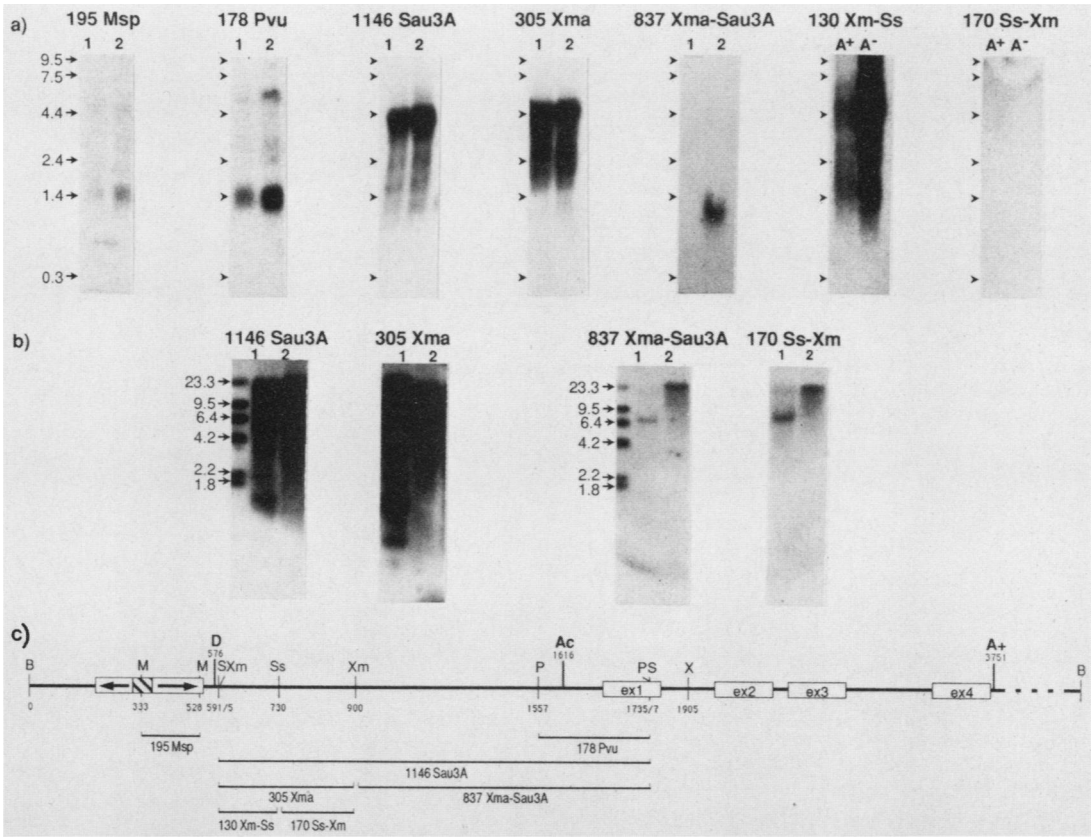

FIG. 1. Northern and Southern blot analysis of the human c-Ha-*ras* gene. The line drawing (c) depicts the 6.4-kb *Bam*HI fragment containing the human c-Ha-*ras*1 gene and flanking sequences. Positions of the donor (D) and acceptor (Ac) splice sites (6, 24) and the polyadenylation site (A+) are indicated by bold vertical lines. Coding exons 1 through 4 (6, 24) are labeled. Open rectangles represent the c-Ha-*ras* direct and reverse promoters (20); direction of transcription is indicated by horizontal arrows. The c-Ha-*ras* up-regulatory element located between the direct and reverse promoters (20) is represented by a hatched rectangle. The probes used for Northern and Southern blot analyses are shown below the line drawing. Restriction enzyme sites: B, *Bam*HI; P, *Pvu*II; M, *Msp*I; X, *Xba*I; S, *Sau*3A; Xm, *Xma*III; Ss, *Sst*II. (a) Northern blot analysis. A 10-μg sample of total or polyadenylated RNA from EJ bladder carcinoma cells (23) (lane 1) or K562 cells (26) (lane 2) was analyzed, using a series of probes derived from the c-Ha-*ras* oncogene. Probes are indicated above each panel and are labeled as in panel c. Total RNA (8) and polyadenylated RNA (10) were isolated from EJ bladder carcinoma cells or from K562 cells. Purified RNA was electrophoresed through 1% formamide denaturing gels, transferred to nitrocellulose (27), and hybridized to α-^{32}P-labeled probes (25) at 42°C overnight. Filters were washed to a final stringency of 0.1% sodium dodecyl sulfate–0.1× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C for 1 h. Autoradiography was for 16 h at −70°C with intensifying screens. Markers are provided by an RNA ladder and are represented by arrows to the left of each autoradiograph. (b) Southern blot analysis. A 10-μg sample of DNA from EJ cells was digested to completion with *Bam*HI (lane 1) or *Eco*RI (lane 2) and analyzed by using probes derived from intron −1 of the c-Ha-*ras* gene. Probes are labeled above each panel as in panel c. Purified genomic DNA from EJ cells was digested to completion with *Bam*HI or *Eco*RI and electrophoresed through 1% agarose. Fractionated DNA was transferred to nitrocellulose (27) and hybridized to α-^{32}P-labeled probes at 65°C overnight (25). Filters were washed to a final stringency of 0.1% sodium dodecyl sulfate–0.1× SSC at 65°C and autoradiographed for 16 h at −70°C with intensifying screens. Markers are provided by *Hin*dIII fragments of λ and are represented by arrows to the left of each autoradiograph.

imately 40-fold less than that produced by the powerful herpesvirus IE promoter (Fig. 2b).

Locations of transcription initiation sites within the repetitive intron element were determined by S1 analysis of RNA (3, 29) from Cos 7 cells after transfection with 460D-CAT and 460R-CAT (Fig. 3). Total RNA was prepared 48 h after transfection, and 20 μg was hybridized in probe excess at 57°C to a single-stranded *Hin*dIII-*Pvu*II fragment derived from either 460D-CAT or 460R-CAT. In the direct orientation, at least 14 initiation sites were detected within the 460-bp *Sau*3A-*Sac*I promoter fragment. Four initiation sites were found to be located within the highly repetitive region at positions +40, +80, +120, and +130 relative to D, and a run of 10 initiation sites was found outside the repetitive element between positions +360 to +460 relative to D. In the reverse orientation, 10 initiation sites were detectable, 5 within the repetitive element at positions +30, +60, +120, +125, and +130 relative to D and 5 at positions +170, +200, +250, +270, and +300. These sites have been confirmed by using a range of S1 digestion conditions and the independent technique of primer extension analysis (not shown).

Transcription terminator within the c-Has-*ras* intron −1 repetitive element. The highly repetitive element coincides with a 150-bp region between +35 and +180 relative to D, which has previously been shown to significantly reduce the transformation efficiency of the c-Ha-*ras* oncogene (17, 18). We speculated that such an effect on transformation efficiency could be achieved if the repetitive element functions as a transcription terminator. Such a mechanism occurs in the c-*myc* (2, 9, 22), c-*myb* (1), and c-*fos* (11) oncogenes,

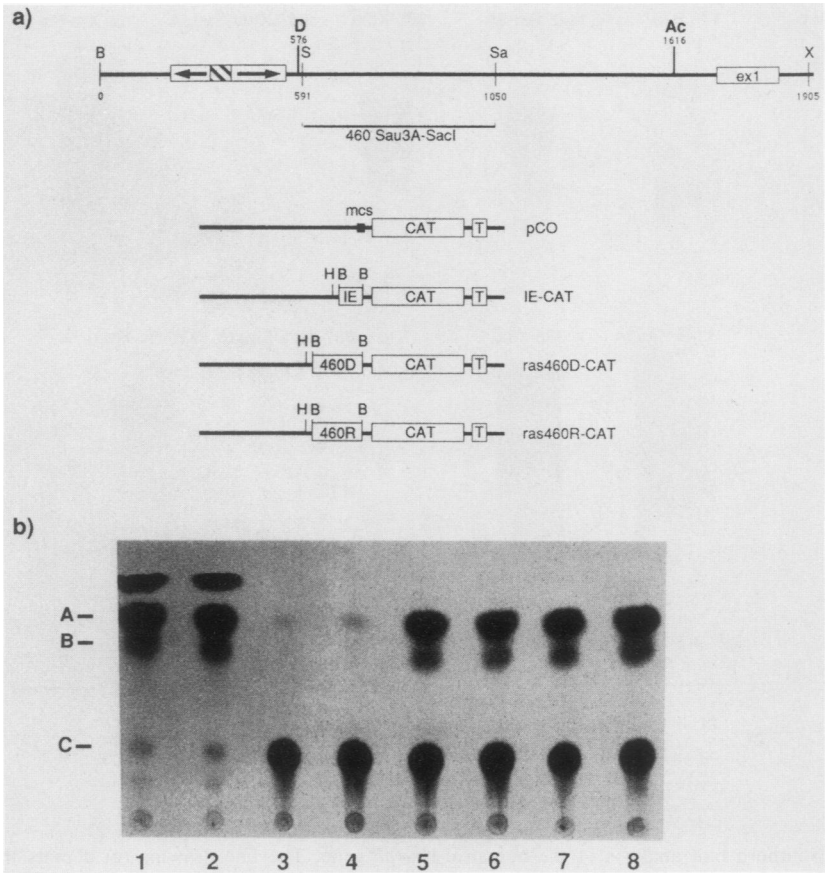

FIG. 2. Analysis of the c-Ha-*ras* repetitive intron promoter. (a) Constructs. The line drawing represents a *Bam*HI-*Xba*I fragment containing the c-Ha-*ras* bidirectional promoter, intron −1, and exon 1. The donor (D) and acceptor (Ac) splice sites are indicated as in Fig. 1. Restriction sites are as in Fig. 1. Sa, *Sac*I. The promoterless CAT construct pCO contains the pUC9 multiple cloning site (mcs), the CAT gene, and a herpes simplex virus type 2 IE gene terminator (T) at the 3′ end of the CAT gene (12, 20). IE-CAT contains the herpesvirus IE promoter inserted by *Bam*HI linkers into the *Bam*HI site of pCO (20). A 460-bp *Sau*3A-*Sac*I fragment, derived from the 5′ end of intron −1 (see line drawing), was inserted in both orientations into the *Bam*HI site of pCO by means of *Bam*HI linkers to generate 460D-CAT and 460R-CAT. D, Direct orientation; R, reverse orientation. (b) CAT analysis. Increasing amounts (from 0.1 to 20 μg per 75-cm^2 flask) of each CAT construct were introduced into Cos 7 cells (14) by calcium phosphate precipitation (31). At 48 h after transfection, cells were harvested and CAT assays were performed on cell lysates as described by Gorman et al. (15). Lanes: 1 and 2, IE-CAT (0.5 μg); 3 and 4, pCO (10 μg); 5 and 6, 460D-CAT (10 μg); 7 and 8, 460R-CAT (10 μg). A, B, and C indicate positions of the 3-acetylchloramphenicol, the 1-acetylchloramphenicol, and the unacetylated [^{14}C]chloramphenicol products, respectively, of CAT activity.

which are regulated by terminator elements within the first exon. To test this hypothesis, we inserted the herpesvirus IE promoter and a 195-bp *Msp*I fragment containing the c-Ha-*ras* direct promoter (Fig. 1) into the *Hin*dIII site of 460D-CAT and 460R-CAT. A 0.5-μg sample of each construct was introduced into Cos 7 cells, and CAT activity was measured 48 h later. This concentration of input DNA was chosen because CAT activity of the IE-CAT construct is maximal (Fig. 2) and background CAT activity from the repetitive element is low. Insertion of the repetitive intron fragment between the IE promoter and CAT in the construct IE-460D-CAT was associated with an eightfold reduction in CAT activity compared with IE-CAT (Fig. 4a and b). This reduction in CAT activity was not found when the 460-bp fragment was inserted in the reverse orientation between the IE promoter and CAT. Only a marginal reduction in CAT activity was found when the repetitive element was inserted between the 195-bp *Msp*I c-Ha-*ras* promoter fragment and the CAT gene.

Reduction in CAT activity by the 460-bp repetitive element could be due to (i) reduction in activity of the upstream promoter, (ii) destabilization of transcripts derived from the upstream promoter, or (iii) termination of these transcripts. To distinguish between these possibilities, we analyzed transcription of the CAT constructs in a nuclear run-on assay (Fig. 4c). Cos 7 cells were transfected with 0.5 μg of constructs IE-CAT, IE-460D-CAT, and pCO per flask. Nuclei were prepared and labeled with [α-^{32}P]dUTP (16, 19). RNA was purified and hybridized to (i) a 200-bp fragment containing the IE promoter, (ii) a 300-bp *Xma*III fragment containing the repetitive element (Fig. 1), (iii) a 200-bp control pBR322 vector sequence, and (iv) a 140-bp *Bam*HI-*Pvu*II fragment containing the CAT gene. The four probes were designed to be of approximately equal length to ensure comparable hybridization signals. The IE transcription initiation site is located 135 bp from the 3′ boundary of the IE fragment (12); thus, the lengths of labeled transcripts hybridizing to the IE promoter and CAT probes are almost identical. Hybridization to the IE, CAT, and pBR322 fragments was carried out in probe excess, determined empirically. This concentration of immobilized probe (250 ng per spot) was also used for the RAS probe. Since this probe is

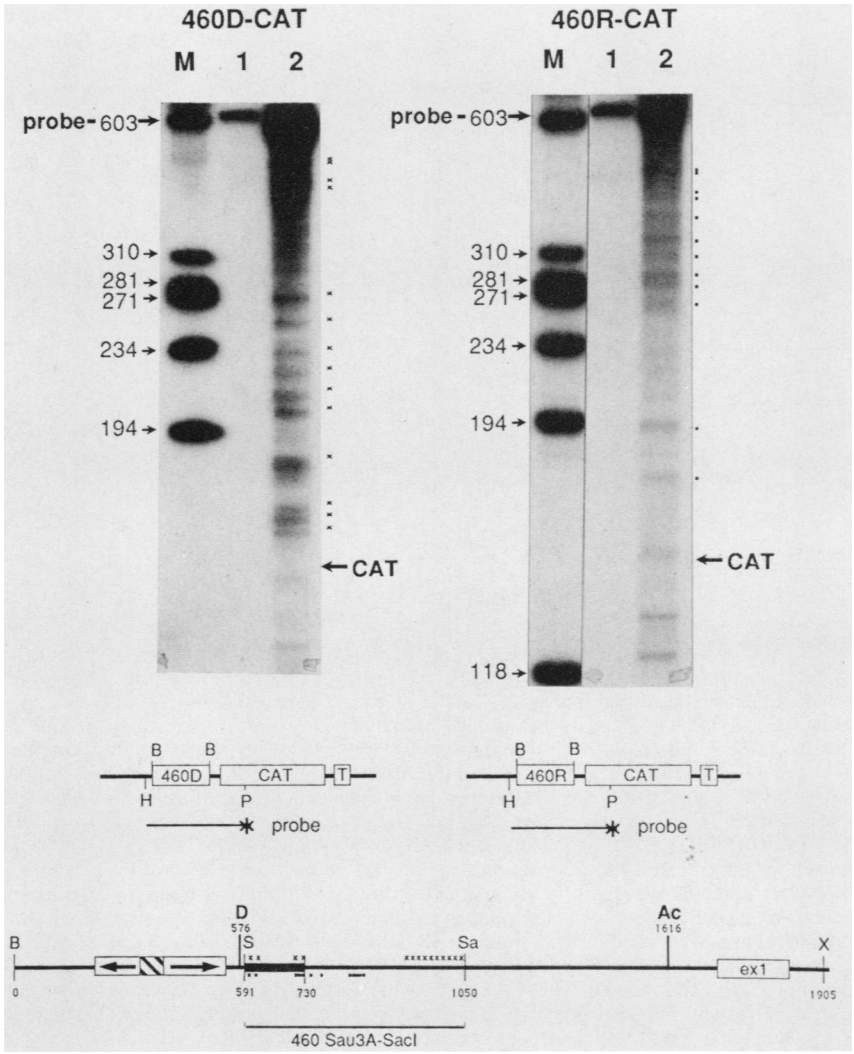

FIG. 3. S1 analysis of the c-Ha-*ras* intron −1 promoter. Cos 7 cells were transfected with 460D- and 460R-CAT and pCO (10 μg per 75-cm^2 flask). At 48 h after transfection, total RNA was prepared, and 20 μg was hybridized in probe excess at 57°C for 16 h to a single-stranded *Hin*dIII-*Pvu*II fragment spanning the 460-bp c-Ha-*ras* repetitive element and the CAT gene (see line drawing). The probe was 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase and strand separated on polyacrylamide (21). Hybrids were digested at 37°C for 1.5 h with 1,000 U of S1 nuclease (Boehringer Mannheim Biochemicals), and digestion products were separated on 6% denaturing polyacrylamide gels (3, 20, 29). Autoradiography was for 36 h at −70°C with an intensifying screen. Lanes: 1, pCO; 2, 460D-CAT or 460R-CAT. Markers (M) are provided by *Hae*III fragments of φX174. Initiation sites are represented by crosses (460D-CAT) or filled circles (460R-CAT) to the right of the appropriate autoradiograph. Initiation sites are also indicated on the line drawing positioned relative to the 130-bp repetitive element, which is indicated by a thickened line.

homologous to repetitive RNA in Cos 7 cells (Fig. 4c, pCO lane), hybridization in this case was not in probe excess and therefore the signal obtained does not accurately reflect cellular levels of RNA species homologous to this fragment. This has no direct bearing on the assay since the relevant comparison is between the IE, pBR322, and CAT probes. When the 460-bp element was inserted, in the direct orientation, between the IE promoter and the CAT gene, transcription from the CAT gene fragment was approximately 10-fold less than from the IE promoter fragment. This result was accurately quantitated by counting the excised spots. In the absence of the 460-bp element, levels of transcription from the IE promoter fragment and the CAT gene were equivalent, strongly suggesting that reduction in CAT activity was the result of premature termination or pausing of IE transcripts within the 460-bp repetitive element.

It is unknown when and if the c-Ha-*ras* repetitive intron element is normally transcribed. However, transcripts originating within intron −1 and extending toward the gene may contribute to mature c-Ha-*ras* mRNA. This view is supported by the consistent finding that lower levels of the 1,400-bp message are detected by probes derived from exon −1 than by probes derived from the coding exon 1 (Fig. 1a).

The significance of the c-Ha-*ras* intron −1 repetitive terminator element for normal regulation of the c-Ha-*ras* gene is unclear. The presence of a transcription terminator within intron −1 may provide a rapid and reversible mechanism by which cell cycle-dependent changes in c-Ha-*ras* expression (5) are achieved. Similar reversible transcriptional blocks have been found to regulate expression of the c-*myc* (2, 9, 22), c-*myb* (1), and c-*fos* (11) genes, and we have recently identified a transcription terminator within the Alu

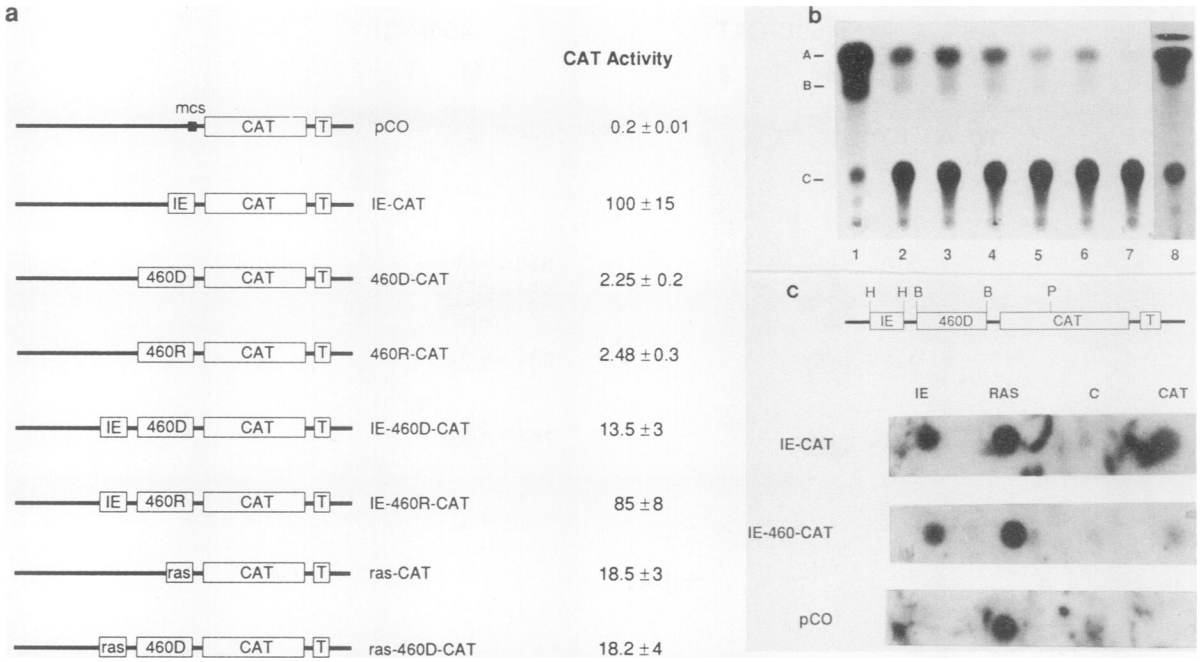

FIG. 4. Demonstration of a transcriptional block within the c-Ha-*ras* intron −1 repetitive element. (a) Summary of CAT activity. pCO, IE-CAT, 460D-CAT, and 460R-CAT are described in Fig. 2. The IE promoter (derived from IE-CAT) and the c-Ha-*ras* direct promoter (195-bp *Msp*I fragment shown in Fig. 1) were inserted by means of *Hin*dIII linkers into the *Hin*dIII site of 460D-CAT and 460R-CAT. A 0.5-μg sample of each construct was transfected into Cos 7 cells as described in Fig. 2; 48 h later, CAT activity was measured (15). Spots were cut out of the thin-layer plates and measured in a scintillation counter. Conversion of [^{14}C]chloramphenicol into acetylated products was calculated, and IE-CAT was given an arbitrary value of 100. Each value is the mean of at least three separate experiments using at least two independent preparations of DNA. (b) CAT activity. Constructs and transfection conditions were as for panel a. Lanes: 1, IE-CAT; 2, IE-460D-CAT; 3, ras-CAT; 4, ras460D-CAT; 5, 460D-CAT; 6, 460R-CAT; 7, pCO; 8, IE-460R-CAT. A, B, and C are as in Fig. 2b. (c) Nuclear run-on assays. Constructs used for the transfections and run-on reactions are shown to the left of the autoradiographs and are described in panel a and in Fig. 2a. IE-460-CAT contains the repetitive element in the direct orientation between the IE promoter and the CAT gene. The DNA probes used for hybridization of the labeled RNAs (250 ng per spot) are shown in the line drawing above the autoradiographs. IE is the 200-bp *Hin*dIII fragment containing the herpesvirus IE promoter. RAS is the 300-bp *Xma*III fragment (see Fig. 1c) containing the repetitive element. C is a 200-bp negative pBR322 control. CAT is a 140-bp *Bam*HI-*Pvu*II fragment containing the 5′ end of the CAT gene. Cos 7 cells were transfected with 0.5 μg of pCO, IE-CAT, and IE-460D-CAT per 75-cm^2 flask as described above; 48 h later, nuclei were prepared exactly as described by Groudine et al. (16), and approximately 10^7 nuclei were suspended in nuclear freezing buffer. The run-on reaction was performed as described by Linial et al. (19). DNA samples were fixed onto nitrocellulose filters in 6× SSC and prehybridized for 24 h at 42°C in hybridization buffer consisting of 10 mM TES, 0.3 M NaCl, 0.5% sodium dodecyl sulfate, 10 mM EDTA, 1× Denhardt solution, and 0.5 mg of salmon sperm DNA per ml. ^{32}P-labeled RNA was hybridized to 100, 250, and 500 ng of IE, pBR322, and CAT probes to empirically establish probe excess. Hybridization was at 42°C for 24 h, and the filters were washed to a final stringency of 0.1× SSC–0.1% sodium dodecyl sulfate at 60°C for 30 min and autoradiographed at −70°C for 36 h.

repetitive element 2.2 kb upstream of the human ε-globin gene. This element up regulates the gene by blocking transcriptional interference from a far upstream promoter (32) and, like the c-Ha-*ras* element, is highly repetitive and transcriptionally active.

An additional function for the intron −1 terminator element is suggested by the finding that this element limits transcription from the powerful herpes simplex virus IE promoter into the coding region of the gene. The repetitive element described in this study coincides with a 150-bp sequence previously shown to down regulate the transforming efficiency of the c-Ha-*ras* oncogene (17, 18). Since herpes simplex virus IE transcripts are terminated by this element, we suggest that the repetitive element influences the transformation efficiency of c-Ha-*ras* by regulating the number of polymerase molecules extending into the coding region of the gene. Such a mechanism would be of particular significance after viral insertion upstream of the gene (30) or under circumstances such as enhancer translocation whereby transcription from the normal c-Ha-*ras* promoter would be elevated (20). The control afforded by this transcriptional "valve" may be a powerful protection against the carcinogenic effects of elevated expression of the c-Ha-*ras* gene, and we predict that mutations within this region will be associated with the progression of certain tumors.

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