Chromatin structure of the promoter region of the human c-K-ras gene

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ABSTRACT

The chromatin structure of the human c-K-ras gene has been investigated in various cultured normal and tumor human cells and in a rat cell line transformed with the human oncogene. The promoter region is hypersensitive to DNase I, micrococcal nuclease, endogenous nucleases and to S1 nuclease in supercoiled plasmids. This hypersensitive region is present in the different cell types analyzed and both normal and mutant alleles exhibit similar general sensitivity to DNase I digestion in the same tumor cells. However, the 5' more distal DNase I hypersensitive site, which is coincident with a region of the gene containing sequence homologies with known enhancers, exhibits variable sensitivity which appears to be higher in the tumor than in the normal and in the human than in the rat cells which we have analyzed. These data suggest the presence of specific factors interacting with the promoter sequences and delimits the transcription unit of the c-K-ras locus.

INTRODUCTION

Chromatin structure of the upstream regions of eukaryotic genes is of interest because these regions contain DNA sequences involved in controlling gene expression and the chromatin structure may be an aspect of this control. Special sites have been recognized in chromatin that are very sensitive to digestion by various nucleases. These hypersensitive sites (HS), which correspond to double strand cuts of DNA in the chromatin fiber have been frequently mapped at the 5' end of numerous genes, coincident with DNA sequences important for gene expression and which bind regulatory proteins. Most often deoxyribonuclease I (DNase I) has been used to study these hypersensitive sites, but HS have been also found with undefined endogenous nucleases, DNase II, micrococcal nuclease and S1 nuclease (for a recent review see ref. 1). The presence of DNase I HS at the 5' end of genes is particularly well correlated with their transcriptional activity (2-9). The nature of the HS is not known, although studies with simian virus 40 (SV40) minichromosomes and the chicken beta globin gene indicate that DNase I HS correspond to regions of

chromatin free of nucleosomes (10,11). Alterations in DNA secondary structure have been proposed to occur in the vicinity of HS (12-14). In addition DNase I hypersensitivity can be conferred by specific protein binding, as demonstrated in a reconstituted system (11). Preferential binding of specific cellular proteins in vivo to sequences within a hypersensitive site has been demonstrated in the *Drosophila* heat-shock gene hsp82 (15) and in the immunoglobulin gene enhancer region (16).

Ras genes represent a family of highly conserved genes in eukaryotic cells encoding structurally related proteins (p21) of unknown function which nevertheless possess oncogenic potential in established rodent cell lines (reviewed in 17). To date, three human ras genes have been characterized, c-H-ras, c-K-ras and N-ras that have been found in a significant percentage of human tumors activated by somatic mutations of their corresponding normal proto-oncogenes (reviewed in 18). The c-K-ras is the gene most often activated in solid human tumors as assayed by the NIH3T3 DNA transfection assay (18). In our studies we have cloned the c-K-ras oncogene from two human lung carcinomas (19), their activating mutations have been determined (19,20), and the oncogene promoter region has been sequenced and characterized by deletion mutagenesis and DNA transfection experiments (21).

Ras genes are expressed at low levels in most tissues (22) and thus belong to the class of housekeeping genes. However, very little is known about the regulation of ras gene expression. As a step towards the understanding of possible relationships between chromatin organization and control of ras gene expression, we have analyzed the chromatin structure of the human c-K-ras gene. We report the presence of DNase I, micrococcal nuclease and endogenous nuclease(s) hypersensitive sites at the promoter region of the gene and discuss the possible functional significance of the nucleotide sequences that underlie these HS.

METHODS

Cell cultures and nuclei preparation

Calu-1, SK-LU-1 and PR371 are human cell lines derived from lung carcinomas, and their origins have been described before (19). 143 is a thymidine kinase deficient cell line derived from a human osteosarcoma and its origin has been previously described (23). Human keratinocytes were a kind gift of Dr. Lorne Teichmann. Human normal fibroblasts were obtained from newborn foreskin as described (24). 1HT1 and 1BT1 are rat cell clones

transformed by the human c-K-ras oncogene of the Calu-1 lung carcinoma cell line and their origins have been described before (25).

Cells were grown to semiconfluency in culture dishes and washed once with PBS prior to nuclear purification. Nuclei were isolated as described (26) with slight modifications. To each plate, 2ml of lysis buffer (10mM Tris-HCl pH 7.4, 1mM $MgCl_2$, 10mM NaCl, 1mM PMSF, 0.5% Nonidet P40) were added and cellular lysis was allowed to proceed for 10 min at room temperature. All subsequent steps were performed at 4°C. The lysed cell suspension was removed from plates and homogenized with a teflon pestle type B Dounce homogenizer. Cellular lysis and nuclear aggregation was controlled by microscopic observation during homogenization. The lysate was centrifuged through 25mM Tris-HCl pH 7.4, 5mM $MgCl_2$ and 250mM sucrose at 500g for 5 min. Nuclear pellets were washed once and resuspended in the appropriate nuclease digestion buffers at a DNA concentration of 0.5-1 mg/ml.

Nuclease digestions

Increasing amounts of DNase I (Worthington) in 40 μ l of 5mM $CaCl_2$ and 1mM $MgCl_2$ were added to 0.5 ml aliquots of nuclei resuspended in 15mM Tris-HCl pH 7.4, 60mM KCl, 15mM NaCl, 0.5mM DTT, 250mM sucrose, 0.05mM $CaCl_2$ and 3mM $MgCl_2$. Nuclei were digested for 3 min at room temperature and the reactions were stopped by the addition of 50 μ l of 5% SDS and 100 mM EDTA. Each sample was then digested with 50 μ l of proteinase K (5 mg/ml) at 37°C overnight. DNA was subsequently isolated by repeated organic extractions and ethanol precipitations.

For micrococcal nuclease digestions, nuclei were resuspended in 10mM Tris-HCl pH 7.4, 0.35M sucrose, 25mM KCl, 100mM NaCl, 5mM $MgCl_2$ and 0.5mM $CaCl_2$ and digested at 37°C for 5 min in 0.5 ml aliquots with 1-50 units of enzyme (Worthington). Digestions were stopped and DNA deproteinized and extracted as described above. Naked DNA control digestions were performed in the same conditions as the nuclear chromatin, except at lower enzyme concentrations.

Endogenous nuclease activity was analyzed by autodigesting 0.5 ml aliquots of nuclear preparations at 37°C for 5 min in S1 digestion buffer (30mM sodium acetate pH 4.5, 3mM $ZnCl_2$, 30mM NaCl, 0.2mM EDTA).

To test the sensitivity to S1 nuclease of c-K-ras sequences in supercoiled plasmids, 15 μ g of CsCl purified plasmid DNA were digested with 1-100 units of S1 nuclease (Boehringer) in 25 μ l of S1 digestion buffer containing 300mM NaCl for 30 min at 37°C. Reactions were stopped by

Figure 1 Localization of DNase I HS on the 5' flanking sequences of the c-K-ras gene from cultured normal keratinocytes (A) and fibroblasts (B) and from tumor 143 (C) and PR371 (D,top) human cells. Isolated nuclei were incubated with increasing amounts of DNase I (indicated as units

at the top). Purified nuclear DNA was then digested with EcoRI (A-C) or PstI (D), electrophoresed through 1% agarose gels and blot transferred to nitrocellulose filters. (E): Purified, total cellular DNA from PR371 cells was digested with increasing amounts of DNase I in the same experimental conditions as the nuclei digestions. The digested DNA was then reextracted with phenol/chloroform, ethanol precipitated, digested with EcoRI and processed as before. Lane 0: no DNase I added. Lanes 1-4: 0.01, 0.02, 0.04 and 0.08 units of DNase I, respectively. Lane N is the same DNase I treated nuclear sample as lane 16 of panel D, digested with EcoRI. The position of HS (1, 2 and 3) is indicated in the map at the right of panel D. The restriction endonuclease sites are: B: BglII; C: ClaI; P: PstI; R: EcoRI. The solid bar indicates the 0.4 Kb EcoRI-BglII fragment present in the plasmid used as radioactive probe. The nitrocellulose filter of panel D (top), was rehybridized with ^{32} P-labeled pL-R2.4 (bottom) to analyze the DNase I sensitivity of the c-K-ras gene 3' end region. The open rectangle indicates the 5' end untranslated exon (41). The numbers to the left indicates the position and size (in Kb) of the lambda HindIII fragments used as molecular weight markers (lanes M).

adding EDTA to a concentration of 50 mM and DNA was deproteinized and extracted as described above.

Southern blot hybridizations

Fifteen to 30 μ g of DNA were digested with the appropriate restriction endonucleases (Bethesda Research Laboratories) and electrophoresed through agarose gels for 15 hours at 2 volt/cm. The DNA was transferred to BA85 Schleicher and Schuell nitrocellulose membranes or to Amersham Hybond-N nylon filters and hybridized to DNA or RNA probes. For DNA probes hybridization conditions were essentially as described (19) but in the presence of 10% dextran sulfate (Pharmacia). For RNA probes hybridizations were performed at 69°C in 5XSSC, 50mM NaH_2PO_4 and 0.05 mg/ml salmon DNA, in the presence of 40% formamide and 10% dextran sulfate. Prior to hybridization filters were incubated for 6-8 h in the same conditions (without probe) but in the presence of 1XDenhardt. Filters were autoradiographed for 1 to 4 days on Kodak XAR-5 film, using intensifying screens (Cronex-Dupont) at -70 °C. HindIII digests of lambda phage DNA and HaeIII digests of OX174 DNA were routinely used as molecular size markers.

Plasmids and probes

pKRBg0.4 is a derivative of pKRSm2.6 (24) and contains the 5' distal 0.4 Kb EcoRI-BglII fragment of the PR371 c-K-ras oncogene derived from λ LD-H15 (19). Plasmids pL-R2.4, pL-RH0.9 (19), and pKRBg0.4 were ^{32} P-labeled by nick-translation as described (19). RNA probes were synthesized as described (24) using pKRP2.8, a plasmid containing the 2.8 Kb EcoRI-PstI fragment of the PR310 c-K-ras oncogene from λ LN-H16 (19), linearized at the ClaI site as template for in vitro transcription.

RESULTS

DNase I hypersensitive sites in the human c-K-ras gene promoter region.

The chromatin structure of the human c-K-ras gene was investigated in several cultured human cells by analyzing its sensitivity to digestion by DNase I using the indirect labelling method (3). Digestion of isolated nuclei with increasing amounts of enzyme revealed the presence of a DNase I hypersensitive region as observed from the 5' end of the gene, using pKRBg0.4 as radioactive probe (Figure 1). This hypersensitive region, located immediately upstream of the 5' end untranslated exon, was observed in all cells tested including normal cultured keratinocytes and fibroblasts (Figure 1 panels A and B) and tumor cells from the osteosarcoma 143 and the lung carcinoma PR371 cell lines (Figure 1 panels C and D,top). The sensitivity to DNase I digestion of this region was specific of the native chromatin of the c-K-ras gene because it was absent in control experiments performed by digesting with DNase I purified DNA from the same cells (Figure 1E). However, some differences in the structure of this hypersensitive region were also apparent between these cells. In 143 and PR371 tumor cells, the DNase I hypersensitive region could be resolved into several distinct HS (Figure 1 panels C and D,top). A distal site to the 5' noncoding exon (HS 1) and a proximal region that appeared as a broad band but that could be resolved into two distinct sites (HS 2 and 3) in other experiments (see below). In normal keratinocytes and fibroblasts, while the bands corresponding to HS 2 and 3 were present, the band corresponding to HS 1 could not be detected (Figure 1A) or was very faint (Figure 1B).

Because the differences in the relative sensitivity of HS 1 were evident at comparable DNase I digestion levels of the original restriction endonuclease DNA fragments (the 4.6 Kb EcoRI fragments in panels A, B and C, and the 9.5 and the 3.5 Kb PstI fragments in panel D,top) and because the amounts of DNA loaded in each lane were essentially the same, these differences are unlikely due to variations in the extent of digestion by DNase I in the different nuclear preparations. For instance, in lane 40 of panel B, approximately 90% of the original EcoRI fragment was digested by DNase I (compare the intensity of this band with that of lane 0) and yet, only traces of HS 1 were present. However, lanes 4 and 8 of panel D show that although the original PstI fragments were only slightly digested (compare with lane 0), HS 1 was already clearly visible. Similar differences in the relative sensitivity to DNase I digestion of HS 1 were

observed in repeated experiments using the same cultured cells and the same mapping strategy for HS.

In the experiment of Figure 1D (top), nuclear DNA from PR371 cells was digested with PstI after DNase I treatment. PR371 is a tumor cell line which contains both normal and mutant c-K-ras alleles and we have shown that the mutant allele is amplified relative to the normal (24, and see Fig. 1D,top). Because the presence of a PstI polymorphic site (indicated with an asterisk at the map of Figure 1D), digestion with this enzyme and hybridization with the pKRBg0.4 probe generates two bands of 3.5 and 9.5 Kb corresponding to the PstI fragments from the normal and mutant c-K-ras alleles, respectively. Both fragments disappeared at similar rates with increasing amounts of DNase I (Figure 1D,top). The same filter was rehybridized with the 2.4 Kb 3' end EcoRI fragment of the c-K-ras gene (see Figure 7) as radioactive probe. This experiment (Figure 1D,bottom), revealed the absence of DNase I hypersensitive sites at the 3' end of the c-K-ras gene in PR371 tumor cells and the 4.4 kb PstI fragment corresponding to the 3' end of the gene appeared slightly more resistant to DNase I digestion than the 5' end PstI fragments (Figure 1D,bottom). These results indicate that there are not obvious differences in the general sensitivity to DNase I digestion at the 5' end region of both normal and mutant c-K-ras alleles in PR371 tumor cells. Similar results were obtained using SK-LU-1, another human lung carcinoma cell line containing the same PstI polymorphism (data not shown).

The middle region of the c-K-ras gene was also analyzed in similar manner using pL-RH0.9 (see Figure 7) as radioactive probe but no other DNase I HS were found (data not shown).

Micrococcal nuclease cleavage sites on the 5' flanking sequences of the human c-K-ras gene.

Micrococcal nuclease was used to probe the chromatin structure of the c-K-ras gene. This enzyme differs from DNase I in its mode of nucleolytic cleavage (27) and thus provides complementary structural information. The presence of micrococcal nuclease cleavage sites on the 5' flanking sequences of the c-K-ras gene from PR371 tumor cells was analyzed in a similar manner to the DNase I HS mapping experiments, using pKRBg0.4 as probe (Figure 2A). When no micrococcal nuclease was added to the nuclear preparation (lane 0) only the band corresponding to the 4.6 Kb EcoRI c-K-ras fragment was observed, indicating the absence of endogenous nuclease activities under these assay conditions. Upon addition of enzyme,

Figure 2 Micrococcal nuclease cleavage sites on the 5' end c-K-ras sequences of PR371 cells. (A) Nuclei were incubated with increasing amounts of enzyme (indicated as units at the top). (B) Purified total cellular DNA from PR371 cells was also digested in the same conditions with increasing amounts of micrococcal nuclease (indicated as units at the top). Lane N is the same micrococcal nuclease treated nuclear sample as lane 6 of panel A. Nuclease treated DNA was then purified, digested with EcoRI and analyzed as described in Figure 1. The position of micrococcal nuclease HS (I-III) is indicated in the map at the right of panel A. The symbols are as in Figure 1.

a distinct set of subbands appeared. Three sharp bands (I,II,and III) and a slower migrating broad band were observed, indicating the presence of four sensitive regions to this nuclease within the explored oncogene fragment. Control experiments performed at identical resolution with purified DNA yielded a different pattern (Figure 2B). Thus, while the broad band was also present, HSII and III were absent. A faint band close to the position of HS I was also observed. Therefore, HS II and III seem

Figure 3 (A) Comparative analysis of HS for different nucleases in the c-K-ras gene from LHT1 rat transformant cells. Isolated nuclei were autodigested in S1 buffer conditions (lane 1) or incubated with 30 or 45 units of DNase I (lanes 2 and 3), or with 10 or 5 units of micrococcal nuclease (lanes 4 and 5). (B) Comparative analysis of DNase I HS in the c-K-ras gene from LHT1 (lane 1) and PR371 (lanes 2 and 3) cells. Nuclei were incubated with 14, 5 and 0 units of DNase I (lanes 1-3) respectively. After nuclease digestion, nuclear DNA was purified, digested with EcoRI and analyzed in agarose gels as before (Figure 1). The position of DNase I (1,2 and 3) and micrococcal nuclease (I,II and III) HS is indicated. The symbols are the same as in Figure 1.

to reflect the nucleoprotein structure of c-K-ras DNA in chromatin rather than intrinsically nuclease sensitive sequences in naked DNA (28). HS I also appears chromatin specific or at least shows a clearly increased sensitivity in chromatin compared with naked DNA. On the other hand, the nature of the faint bands visible at higher micrococcal nuclease digestion levels in the bottom part of the film (Figure 2A, lanes 4-15) is not clear. They could originate from nucleosomes placed orderly on the c-K-ras 5' flanking sequences. However, they could also be originated by intrinsic sensitivity to this nuclease of the 5' end distal oncogene sequences, because faint bands were also present at similar positions in control experiments using naked DNA (Figure 2B). Further experiments will be necessary to resolve this question.

Similar experiments performed with human normal fibroblasts and 143 tumor cells and rat fibroblasts of the LHT1 and LBT1 clones revealed HS I-III at the same positions. However, in 143 nuclei the sensitivity of site II was much lower than that of sites I and III (data not shown).

DNase I and micrococcal nuclease HS in the c-K-ras gene of transformed rat fibroblasts.

The presence of DNase I HS on the human c-K-ras gene was also analyzed in a transformed rat cell line (1HT1) and derived cell clones. 1HT1 was generated by transformation of the parental normal rat fibroblast cell line (Rat 4), which is deficient in nuclear thymidine kinase (29), with the human oncogene of the Calu-1 lung carcinoma cell line and cotransformation with the cloned chicken thymidine kinase (tk) gene (30). 1HT1 cells were selected in HAT medium and contain about 10 copies of the human oncogene. 1BT1 is a cell clone derived from 1HT1 by selection in medium containing bromodeoxyuridine (BUDR) which nevertheless still retain both tk and c-K-ras genes but in lower copy number. The number of copies of both tk and c-K-ras genes in 1BT1 was again increased to about 10 copies by back selection in HAT medium. The details of these experiments have been previously described (25).

The chromatin structure of the human c-K-ras gene was analyzed as before in 1HT1 cells (Figure 3) and in 1BT1 cells (Figure 4A) containing low (Figure 4a) or high (Figure 4b) oncogene copy number. The same HS detected in human cells were also detected in 1HT1 cells and at the same positions (Figure 3B). In rat transformants, observed from its 5' end, the bands corresponding to DNase I HS 2 and 3 were strong while the band corresponding to HS 1 was weaker than in PR371 human tumor cells. This difference in intensity was observed even at higher DNase I digestion levels (Figure 4A). Parallel experiments performed with the same DNase I treated nuclei from 1BT1 cells revealed the presence of a DNase I hypersensitive region in the chicken tk gene (indicated by an arrow at the right) (Figure 4B). An endogenous nuclease activity was present in these nuclei which slightly cleaved the gene in the absence of added DNase I, and the HS broadened with increasing amounts of enzyme, in agreement with the previously reported behaviour of the chicken tk gene chromatin to DNase I digestion (31,32).

The original EcoRI 5' end fragment of the c-K-ras gene present in 1BT1 cells was digested by DNase I (Figure 4A) at similar rate to that of the cotransfected tk gene (Figure 4B), regardless of their relative copy numbers. These results indicate that the relative lower sensitivity to DNase I digestion of HS 1 in 1HT1 and 1BT1 cells was not due to incomplete digestion by the enzyme in the transformed rat fibroblasts nuclei preparations. At the same time, these experiments suggest that the

Figure 4 Presence of DNase I HS on the 5' end flanking sequences of the human c-K-ras (A) and the chicken tk (B) genes in transformed rat fibroblasts. Nuclei were prepared from 1BT1 cells grown in BUdR (a, lanes 0-60) or in HAT (b, lanes 0-60) media, and incubated with increasing amounts of DNase I (indicated as units at the top). Purified nuclear DNA was digested with EcoRI (A) or with HindIII (B), electrophoresed through 1% agarose gels and blot transferred to nitrocellulose filters. The filters were hybridized with ^{32} P-labeled pKRBg0.4 (A) or pchtk2 (B), a plasmid containing chicken tk sequences (30). The HS region of the chicken tk gene is indicated by an arrow at the right of panel B. The symbols are the same as in Figure 1.

chromatin structure was the same in the multiple copies of the c-K-ras gene in 1HT1 and derived clones, which is consistent with the direct correlation that we have found between the relative copy number of the c-K-ras oncogene and its relative expression levels in these cells (25).

Mapping of HS in the c-K-ras gene promoter region from their 3' end.

The positions of the DNase I HS on the human c-K-ras gene were mapped with more resolution in 1HT1 cells. The higher copy number of c-K-ras sequences in this cell line facilitated this task. After digestion of nuclei with DNase I, purified DNA was digested with EcoRI and PstI and hybridized to the PstI-ClaI riboprobe (Figure 5A). PstI cleaves downstream of the hypersensitive region, generating subbands of small size and this

Figure 5 Mapping of DNase I (A) and micrococcal nuclease (B) HS in the c-K-ras gene from LHT1 cells. Nuclei were digested with 0, 10 and 14 units of DNase I (A, lanes 1-3) or with 0, 15 and 20 units units of micrococcal nuclease (B, lanes 1-3), respectively. After nuclease digestions, purified DNA was digested with EcoRI and PstI, electrophoresed through 1.5% agarose gels and blot transferred to nylon filters. The position of the micrococcal nuclease HS is indicated in the map at the right. The solid bar indicates the fragment of the gene (PstI-ClaI) corresponding to the ³²P-labeled RNA probe. The open rectangle represents the position of the 5' end untranslated exon. The restriction endonucleases sites are: A: AvaI; C: ClaI; P: PstI; R: EcoRI; S: SstII. The numbers at the left indicate the position and size (in Kb) of the HindIII digests of lambda DNA and HaeIII digests of ϕ X174 DNA used as molecular weight markers (M).

allowed us to map the HS more accurately. Bands corresponding to HS 2 and 3 showed similar intensity, and were mapped at 650 and 450 bp upstream of the 3' PstI site, respectively. DNase I sensitivity decreased between the bands corresponding to these sites. HS 1 was not detected, probably due to the lower sensitivity of this site in LHT1 cells.

As for DNase I, we took advantage of the identity in position of micrococcal nuclease HS I-III between LHT1 and human cell nuclei (Figure 3) to map them with higher resolution in LHT1 cells (Figure 5B). As observed from its 3' end, HS III and II were resolved into two doublets (III₂ and III₁, and II₂ and II₁) located at 390 and 500 bp and at 680 and 720 bp upstream of the 3' PstI site, respectively. Site I was detected as a single band situated 950 bp away from the 3' PstI site. The intensity of the bands corresponding to these sites changed as seen from its 3' end. Now sites III appeared as the strongest.

Figure 6 S1 nuclease cleavage sites of the c-K-ras 5' end sequences in supercoiled plasmids. (A) pSP64 (lanes 1-3) or pKRSm2.6 (lanes 5-7) were digested with 1, 10 and 100 units of S1 nuclease. Digested DNA was electrophoresed through a 1% agarose gel and visualized by ethidium bromide staining. EcoRI linearized pSP64 (lane 4) or pKRSm2.6 (lane 8) were included as controls. The supercoiled bands of both plasmids migrate faster than the linear bands. (B) DNA from samples of lanes 5, 6 and 7 of Figure 4A was purified after S1 digestion, digested with PstI (lanes 1-3) or with PstI and EcoRI (lanes 4-6), electrophoresed through a 1.5% agarose gel and blot transferred to a nylon filter. Blot analysis was as described in the legend of Figure 5.

Sensitivity of the c-K-ras promoter region sequences to S1 nuclease..

In 1HT1 (Figure 3A) and in human cells (data not shown), the same c-K-ras sequences hypersensitive to DNase I were also sensitive to an endogenous nuclease that was active in S1 digestion conditions. After a short incubation of nuclei in S1 buffer, a broad band comigrating with the bands corresponding to DNase I HS 2 and 3 was detected (compare lanes 1, 2 and 3). Addition of S1 nuclease did not change this digestion pattern thus preventing us to study the sensitivity of the c-K-ras 5' end chromatin to S1 nuclease.

However, it has been shown that the same sequences sensitive to S1 *in vivo* are often sensitive to S1 in supercoiled plasmids (12-14). The sensitivity to S1 of pKRSm2.6 was investigated (Figure 6). Within the oncogene fragment present in this plasmid map all the HS described so far. Digestion of pKRSm2.6 with increasing amounts of S1 nuclease resulted in a progressive loss of its supercoiled form and in an increase of its linear and circular forms (Figure 6A, lanes 5-7). The S1 sensitivity of this plasmid was strongly dependent on supercoiling. Digestion with S1 of the

Figure 7 Chromatin organization of the c-K-ras promoter region. The top of the Figure depicts a partial restriction endonuclease map of the human c-K-ras gene. The solid vertical rectangles represent the position of the coding exons with their respective numbers indicated above. The open rectangles indicate the position of the 5' end (β) and 3' end non-coding exons. The bars at the top indicate the gene fragments used as radioactive DNA (solid bars) or RNA (open bar) probes. The restriction endonuclease sites are: A: *Ava*I; B: *Bgl*II; C: *Cla*I; H: *Hind*III; M: *Mst*II; P: *Pst*I; S: *Sst*II; Sc: *Sca*I; Sm: *Sma*I. The *Ava*I-*Sma*I fragment containing the promoter region is represented at higher magnification (bottom). The position of hypersensitive sites to DNase I (HS 1,2 and 3), micrococcal nuclease (HS I, II₁ and II₂, and III₁ and III₂) and to S1 nuclease (S1a) are represented by solid, open and hatched triangles respectively. The 5' end untranslated exon and the region of transcription initiation are represented by solid and broken lines with the arrows indicating the direction of transcription. The position of the hexanucleotide CCGCCC is represented by solid arrowheads indicating their relative polarity. Imperfect sequence homology is indicated by open arrowheads. The position of inverted repeat sequences showing homology to the SV40 core enhancer sequence is indicated by dotted pentagons.

pSP64 vector under the same conditions had much smaller effects on plasmid integrity (Figure 6A, lanes 1-3).

To map the position of the S1 cleavage sites, pKRSm2.6 was digested with increasing amounts of S1 and then with *Pst*I (which cleaves at the polylinker of the cloning vector pSP64) or with *Pst*I and *Eco*RI. The digested plasmid DNA was electrophoresed, blot transferred to a nylon membrane and hybridized with the *Pst*I-*Cla*I riboprobe. One major S1 site (S1a) was detected within the oncogene sequences (Figure 6B) together with other weaker sites in the 5' end distal sequences of the gene and in the plasmid sequences (indicated by arrows at the left of the figure). Site S1a is coincident with the position of DNase I HS 3.

Data on the position of HS for the various nucleases studied are summarized in Figure 7. The HS are mapped indicating their center and width although the detection procedure used is likely to have resulted in an overestimation of their length and an error of ± 30 nucleotides in their mapping.

DISCUSSION

The promoter region of the c-K-ras gene exhibits hypersensitivity to digestion with several nucleases resulting in a pattern of HS that expands for more than 500 bp. These hypersensitive sites are recognized by nucleases of different sequence specificity including DNase I, micrococcal nuclease, an endogenous nuclease that is active in S1 buffer conditions and by S1 nuclease in supercoiled plasmids containing these c-K-ras sequences. All these HS involve DNA sequences which we suspect on the basis of other data to be functionally important.

Both normal and mutant alleles seem to exhibit similar general sensitivity to DNase I digestion in the same tumor cells, and the overall hypersensitivity pattern of the c-K-ras promoter is similar in nuclei from different cell types, supporting the ubiquitous expression of this ras gene. Thus, the proximal hypersensitive region corresponding to DNase I and micrococcal nuclease HS 3 and III which is also recognized by the endogenous nuclease activity and by S1 nuclease in supercoiled plasmids, is present in all cell types analyzed, including normal and tumor human cells of both fibroblastic and epithelial origins and in rat fibroblasts transformed by the human oncogene. This region corresponds to the essential elements of the c-K-ras gene promoter (21).

The observation that these sequences of the c-K-ras gene promoter region are recognized by S1 nuclease in supercoiled plasmids (Figure 6), gives additional support to the hypothesis that those sequences may have a regulatory function. Examples of promoter elements that are hypersensitive to S1 as supercoiled DNA include those of the chicken beta globin gene, Drosophila heat-shock protein 70 and histones genes, human DHFR gene and the SV40 promoter-enhancer region (14,33, reviewed in 13).

The region located between HS 2 (II) and 3 (III) is accessible to digestion by the endogenous activity as well as by DNase I, but at the same time appears relatively resistant to digestion by micrococcal nuclease and although in a lesser extent, by DNase I, suggesting the absence of a typical nucleosome and the presence of interacting

non-histone proteins in this region. The DNA segment corresponding to this region contains a GC rich sequence that resembles the 21 bp repeats of SV40 (34) (Figure 7), and that also shows homology to sequences found in other ubiquitously expressed genes such as the human dihydrofolate reductase (DHFR) gene (35). In SV40, the 21 bp repeats are relatively resistant to digestion by DNase I (5), are flanked by HS in chromatin (36) and bind the transcription factor Spl (37). Furthermore, Spl has been shown to bind and to activate the transcription of the mouse DHFR gene (38) and recently has been shown that Spl also binds to the promoter region of the human c-H-ras gene (39). All these data suggest that the sequences of this region of the c-K-ras gene constitute a functional element of its promoter.

DNase I and micrococcal nuclease HS 1 and I are located in a region of the c-K-ras promoter where we have found (21) sequence similarities with the SV40 enhancer (40). While the relative sensitivity of the micrococcal nuclease site I is very similar in all cells tested (Figures 2 and 3 and data not shown), DNase I HS 1 shows variable relative sensitivity which appears to be higher in the human tumor cells than in the normal human and the transformed rat cells analyzed (Figures 1, 3 and 4). These results and the consistent correlation found between enhancer functionality and the presence of "non-constitutive" DNase I HS located on enhancer sequences (5-8), tempt us to speculate for a possible transcriptional regulatory function of these sequences of the c-K-ras promoter region. Currently, we are testing several fragments of the c-K-ras promoter region for enhancer activity.

In any case our data suggest the presence of specific proteins binding to the c-K-ras 5' distal hypersensitive region. Because the sequences underlying this site are likely the same in all cells studied, the observed DNase I sensitivity differences probably arise from differential binding of protein factors to or near these sequences. Consistent with this hypothesis, we have recently found nuclear protein factors in Hela cells that specifically bind to the c-K-ras gene sequences corresponding to the 5' end distal hypersensitive region, as detected by DNase I footprinting analysis (J.J. and M.P., work in progress).

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