The coding sequences of mouse H2A and H3 histone genes contains a conserved seven nucleotide element that interacts with nuclear factors and is necessary for normal expression

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ABSTRACT

Expression of replication-dependent histone genes of all classes is up-regulated coordinately at the onset of DNA synthesis. The cellular signals involved in coordinate regulation of these genes are not known. Here we report identification of an α element, present within the mouse histone coding region activating sequence (CRAS). We show evidence that this element is present in histone genes from two classes, H2a and H3, in the mouse. This element has two biological functions in histone gene expression, i.e. the element interacts with nuclear proteins in regulation of gene expression, as well as encoding the amino acids of the histone proteins. We present both in vivo and in vitro evidence that interaction of nuclear proteins with this element is required for normal expression. The binding site for nuclear protein(s) has been precisely defined by means of synthetic oligonucleotides, as well as DNase I protection and methylation interference. It is interesting to note that the histone CRAS α element is mutated in a replication-independent H3.3 gene; 5 of 7 nt in the CRAS α box are changed in this gene.

INTRODUCTION

The replication-dependent histone genes belong to a highly conserved gene family. The products encoded by these genes are the basic proteins required to complete nucleosomal packaging of daughter chromosomes after DNA is replicated in the eukaryotic cell. The expression of these histone genes peaks in S phase of the cell cycle (1-4). There are at least two major clusters of histone genes in the mouse, one on chromosome 3 and the other on chromosome 13 (5). Two replication-dependent histone genes, encoding proteins H2a.2 and H3.2, map to chromosome 3 and are among the most highly expressed genes known (13,14).

The promoters of mammalian replication-dependent histone genes are very similar to those of most protein encoding genes, i.e. they contain a proximal TATAA box and may contain CAAT and SP1 elements. Various laboratories have identified histone class-specific promoter elements implicated in the correct regulation of these mouse or human genes in the cell cycle (7-11). For example, an octamer element is required for correct expression of H2b genes (12), but this element is not present in the promoters of histone genes of other classes (H2a, H3 and H4). In fact, no class-specific element has been identified for H3 genes, which coincidentally are among the most highly conserved genes throughout evolution.

The coordinated regulation of mammalian replication-dependent histone genes of all classes is remarkable both for the number of genes of each class that must be up-regulated and for its timing late in G1 in the cell cycle. The cellular players in this regulatory process have not yet been identified. Extensive deletion and mutation studies of histone gene promoters have been remarkably unsuccessful in identifying a common regulatory element involved in control of expression of more than a single class of histone genes, as stated above. Our identification of an element located not in the 5' flanking sequence but in the protein encoding sequence of replication-dependent histone genes of two classes was the first such report (13,14). We identified a coding region activating sequence (CRAS) by in-frame deletions in replication-dependent genes H3.2 and H2a.2. Deletion of the CRAS (110 nt) in either gene caused a 20-fold drop in expression of the H2a or H3 genes when stably transfected into CHO cells (13,14), a much greater effect than is observed in promoter deletion studies of these genes (15).

By stable transfection of histone gene constructs into CHO cells we have previously shown that the H3.2 CRAS restored high expression to a H2a.2 gene with the CRAS deleted (13). We have also shown that the function of this element is independent of position but is orientation dependent by moving the CRAS 700 nt 5' of its native position into the distal H3 promoter (13). These experiments ruled out effects on post-transcriptional processes and showed clearly that the histone gene CRAS is involved in initiation of transcription. Further, we showed that the CRAS from these two replication-dependent genes interacted with common nuclear factors in electrophoretic mobility shift assays (13). Many intragenic elements have been reported for genes transcribed by RNA polymerase II. Most of these elements reside in introns or downstream of the transcription start site in the untranslated sequence (see, for example, 9,16-24). Location of

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The CRAS α element is the first example of a conserved, common role this element may play in correct expression in the cell cycle. An in vitro interaction with nuclear proteins completely abolishes the conserved CRAS α sequence and does not form DNA-protein complexes normally formed upon incubation of the CRAS of two replication-dependent histone genes, that produces the α DNA-protein complexes. Mutation of this target sequence for the nuclear protein(s) involved in control of replication-dependent H3.2 gene expression of the H2a and H3 genes. The evidence presented here regarding its role is five-fold. (i) Nucleic protection analysis of total RNA isolated from pools of stable transfectants shows that deletion of the CRAS α element causes a 4-fold drop in expression of the H3.2 gene in vivo. (ii) Deletion of the CRAS α sequence also causes a total disappearance of the DNA–protein complexes normally formed in vitro upon incubation of the wild-type CRAS fragment with mouse nuclear extract in gel mobility shift assays. (iii) The nucleotides in the H3.2 CRAS α element have been identified by DNase I and methylation interference analyses as the site of DNA–protein interactions that form the CRAS α complexes; these nucleotides constitute one of two 7 bp matches between the CRAS of the H2a.2 and H3.2 genes. (iv) Deletion of the H2a or H3 CRAS conserved α sequence by restriction enzymes ('stairway' analysis) causes identical disappearance of the α complexes in mobility shift assays. (v) An H3.3 gene that is replication independent has 5 out of 7 nt changes in the conserved CRAS α sequence and does not form DNA–protein complexes in binding assays. This is a naturally occurring mutant form of the CRAS α box, the product of H3 gene evolution in the mouse, and implicates the CRAS α box in correct expression of replication-dependent histone genes in the cell cycle.

We further show that directed mutagenesis of the CRAS α box has the same effect on replication-dependent H3.2 gene expression in stable transfectants as does deletion of 24 nt that include the α box sequence. Both the α box mutant gene and the deletion gene show a 4-fold drop in expression when compared with expression of the intact gene. Like deletion of the CRAS α box, mutation completely abolishes the in vitro interaction with nuclear proteins that produces the α DNA–protein complexes. Mutation of this element in a replication-independent H3 gene is suggestive of the role this element may play in correct expression in the cell cycle. The CRAS α element is the first example of a conserved, common element involved in regulation of expression of more than one class of histone genes. This finding supports our hypothesis that sequences within the histone CRAS play a central role in regulation of perhaps all classes of replication-dependent histone genes.

MATERIALS AND METHODS

Oligonucleotide-directed site-specific mutagenesis

A Sall–HindIII restriction fragment, containing the 3′ half of the coding sequence and all of the 3′ flanking sequence, was subcloned from the mouse H3.2-614 gene (5) into pGEM3Zf(+). Oligonucleotide-directed mutagenesis was performed according to the Kunkel method (26). The oligonucleotides were synthesized to direct in-frame deletions within the H3.2 CRAS, each containing a unique EcoRV restriction site (GATATC) to replace the deleted CRAS sequence. The nucleotides deleted or mutated are indicated in Figure 1 by arrows and the numbers above the arrows directly correspond to the mutant gene diagrams at the bottom of Figure 2. Hereafter H3 or H2a will refer to the H3.2 or H2a.2 genes from mouse chromosome 3, unless otherwise noted.

After the deletions were confirmed by sequencing the mutant genes were reconstructed by ligation of the Sall–HindIII deletion mutant into a construct containing the 5′ flanking sequence and the 5′ half of the H3.2 coding sequence (EcoRI–Sall fragment in the appropriate vector) at the Sall site. The deletion mutant H3CRASα was created by alteration of the 7 nt of the H3 CRAS α box by oligonucleotide-directed mutagenesis (26). The CRAS α box was mutated from the native sequence CATGCGG to the sequence TTCTAGA. This mutation was confirmed by sequencing and the gene reconstructed as above.

Cell culture and stable transfections

Chinese hamster ovary (CHO) cells were grown in McCoy's 5A medium supplemented with 10% calf serum, 5% CO2 at 37°C. Pools of stable transfectants were selected after co-transfection of the histone gene of interest with the pSV2neo plasmid by iodine detergent, polybrene and dimethylsulfoxide (27). Selection with the drug G418 was begun 18 h after addition of the gene constructs. Triplicate pools of G418-resistant cells were ready for RNA isolation and analysis 10–14 days after co-transfection of the neomycin resistance and mutant histone genes (15).

RNA isolation and analysis

Cells were harvested at 50% confluence by trypsinization and total RNA was prepared by the hot phenol method (5). The amount of histone mRNA for the gene of interest was quantified by S1 nuclease protection assay (5). Specific 3'-end-labeled probes for each of the H3.2 CRAS deletions were produced. Each deletion plasmid was linearized at the Ncol restriction site, which spans the initiation codon of the H3.2 gene. The ends were filled in with Klenow fragment using [α-32P]dCTP and [α-32P]dATP, then digested with BglII. We determined the specific activities of the probes by loading 125 ng on an agarose gel and excising the labeled fragment, then melting and determining the amount of radioactivity by scintillation counting. Specific activities for all probes ranged between 5 × 105 and 1 × 106 c.p.m./μg. S1 nuclease-protected fragments were separated on 6% polyacrylamide–8 M urea gels. Gels were dried, directly quantified with a Betascope and then autoradiographed for densitometric analysis. Relative expression was determined by calculation of the ratio of...
In vivo expression of the H3.2 CRAS mutants. S1 nuclease protection assays of total RNA isolated from stably transfected CHO cells are shown. Five micrograms of total cellular RNA were hybridized with the specific probe overnight as previously described. The transfected gene is indicated above the lane. Expression of each gene was assayed with the homologous gene as probe (diagrammed beneath the figure). Lane 1 shows expression of the intact H3.2 gene. H3h refers to fragments protected by endogenous hamster mRNAs. The H3h band served as an internal control to standardize for differences in amounts of RNA or probe specific activities. The two H3h bands in lanes 2 and 3 were added together for quantitation (see Results). H3m refers to the band resulting from protection by the specific mouse mRNAs indicated above the lane. All probes were 3'-end-labeled with Klenow fragment at the NcoI site, which spans the ATG codon of these genes. Some lanes were from overexposed film, so that the mouse-specific band would be clearly visible. Results of quantitation are shown in Table 1 (experiment 1).

the endogenous hamster H3 band to the specific mouse H3 band. The ratio obtained for the intact gene was used as 100% and the ratio obtained for the mutant genes was then compared with that of the wild-type.

Nuclear extracts

Mouse myeloma cells were grown in spinner cultures to a density of 5 x 10^6 cells/ml in Dulbecco's modified Eagle's medium, 10% horse serum and 5% CO2. Nuclear extracts were prepared by our modification (13) of the method of Shapiro et al. (28). Aliquots were stored in liquid nitrogen for later use.

DNA-cellulose chromatography

Crude nuclear extract was prepared as described above and applied to DNA-cellulose resin (40 ml bed volume) equilibrated with DC100 buffer (20 mM HEPES, pH 7.9, 0.2 mM EDTA, 0.2 mM EGTA, 100 mM KCl, 20% glycerol, 2 mM DTT). Proteins were eluted with DC400 buffer containing 400 mM KCl. Fractions (4 ml) were collected and aliquots analyzed for CRAS binding activity by electrophoretic mobility shift assay.

Electrophoretic mobility shift assays (EMSA)

The H3.2 CRAS or the H2a.2 CRAS were excised from the appropriate subclone and 3'-end-labeled with [α-32P]dCTP or [α-32P]dATP with the Klenow fragment of DNA polymerase I. The labeled fragment was purified by gel electrophoresis on a 6% native acrylamide gel and eluted from the gel. Labeled fragment (1.5 ng), poly(dl-dC) (0.2 mg/ml final concentration; Pharmacia) and mouse myeloma nuclear extract (6 μg protein/reaction) were incubated at 4°C in binding buffer (final concentration 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol). The total reaction volume (10 μl) was incubated for 20 min and then analyzed by electrophoresis on a 4% native polyacrylamide gel at 200 V (13, 29). Quantitation of radioactivity was obtained directly by analysis of the intact gel with a Betascope (Betagen) or by autoradiography of the gel after drying. Films were scanned with a PDI film scanner (Protein & DNA Imageware Systems).

Synthetic nucleotides utilized in competition experiments were synthesized for the H3.2 CRAS α box and the H3.3 'CRAS α' box with BamHI overhangs, designated by lower case letters. These were: H3.2 CRAS α duplex (including most of the sequence deleted in CRAS4)

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GAGCCGGGAGGACGTCGATAACCACGAAACGTCCTCCGCctagg
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and H3.3 'CRAS α' duplex (the sequence of H3.3 in the α region when aligned to H3.2; refer to Figure 7 for CRAS sequence comparison between the two genes)

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ggatcCTCGGCCGTCATGCGCTGCAGGAGGC

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The sequences used for synthesis of the H3.3 oligonucleotides were obtained from published mouse H3.3 sequences (30-32).

DNase I protection

Binding reactions (50 μl) contained 7.5 ng labeled CRAS fragment, 10 μg poly(dl-dC) (Pharmacia) and 40 μg crude nuclear extract. DNase I (12.5 or 25 ng; Pharmacia) digestions were performed as described by Singh (33). Free and bound probe molecules were separated on a 4% native gel, eluted at 37°C overnight and analyzed on an 8% polyacrylamide–8 M urea sequencing gel.

Methylation interference

Labeled CRAS molecules were partially methylated at G residues with dimethylsulfoxide as described by Siebenlist and Gilbert (34). Labeled probe molecules (0.2 pmol) were methylated in 100 μl total volume for 2 min and precipitated. Binding reactions as described above contained 7.5 ng modified probe. Free and bound molecules were separated as described above for DNase I protection assays and excised from the gel. Molecules were electroeluted from the gel slice and cleaved with 1 M piperidine at 90°C for 15 min. The cleavage products were analyzed on an 8% sequencing gel.
In vivo expression analysis of the H3 CRAS deletion genes appear in Figure 2, lanes 2 and 3 as the lower of the two upper diagrams. Bands resulting from partially digested probe fragments end of the stem-loop of the mRNA molecules, as indicated in the H3.2CRAS la, H3.2CRAS2, H3.2CRAS3 and H3.2CRAS4, to the the mutated mouse genes. These homologous probes map the H3 gene. The probe fragments protected by endogenous hamster constructs and endogenous hamster genes. The probes used for the fragments protected by RNA from both transfected mouse gene of the deletion mutants indicate the expected size of probe genes can be seen in Figure 2. At the bottom of Figure 2 diagrams Results of S1 nuclease protection assays for the CRAS deletion mRNAs up to the point of the CRAS deletion are indicated by H3 SI analysis were homologous to the deletion genes and 3'-end-of the entire CRAS fragments from either replication-dependent nucleotide sequences in Figure 1 by bold type. In-frame deletion of the entire CRAS fragments from either replication-dependent gene caused a 20-fold drop in expression when compared with expression of the intact gene (13,14). In order to determine the exact sequences that contribute to the activation of expression of the H3.2 histone gene we determined expression levels for a set exact sequences that contribute to the activation of expression of the intact gene (13,14). In order to determine the exact sequences that contribute to the activation of expression of the H3.2 histone gene we determined expression levels for a set exact matches of 7 nt are indicated on both the H3 and H2a nucleotide sequences of the H3.2 and H2a.2 CRAS are shown in Figure 1. Two exact matches of 7 nt are indicated on both the H3 and H2a nucleotide sequences in Figure 1 by bold type. In-frame deletion of the entire CRAS fragments from either replication-dependent gene caused a 20-fold drop in expression when compared with expression of the intact gene (13,14). In order to determine the exact sequences that contribute to the activation of expression of the H3.2 histone gene we determined expression levels for a set of in-frame deletions within the H3.2 CRAS. Regions deleted are indicated by 1a, 1b, 2, 3 and 4 in Figure 1. The mutant genes were stably transfected into CHO cells and total mRNA was isolated from pools of neomycin-resistant cells in logarithmic growth.

In vivo expression analysis of the H3 CRAS deletion genes

Results of S1 nuclease protection assays for the CRAS deletion genes can be seen in Figure 2. At the bottom of Figure 2 diagrams of the deletion mutants indicate the expected size of probe fragments protected by RNA from both transfected mouse gene constructs and endogenous hamster genes. The probes used for the S1 analysis were homologous to the deletion genes and 3'-end-labeled at the NcoI site, which is the initiation codon of the H3.2 gene. The probe fragments protected by endogenous hamster mRNAs up to the point of the CRAS deletion are indicated by H3h. H3m indicates the probe fragments protected by transcripts from the mutated mouse genes. These homologous probes map the full-length transcripts of the CRAS deletion genes, labeled H3.2CRAS la, H3.2CRAS2, H3.2CRAS3 and H3.2CRAS4, to the end of the stem-loop of the mRNA molecules, as indicated in the diagrams. Bands resulting from partially digested probe fragments appear in Figure 2, lanes 2 and 3 as the lower of the two upper bands seen in these lanes and are labeled H3h in the figure. These fragments resulted from inefficient digestion of the CRAS1a probe–endogenous hamster mRNA duplex by S1 nuclease in the region of mutation 1a. Gene H3.2CRAS1a has a coding region mutation of only a few nucleotides. If this region of non-homology with the endogenous (hamster) mRNA was not recognized and digested by the nuclease a probe fragment corresponding to the hamster mRNA mapped to the stop codon was observed.

At least three pools of stable transfectants were analyzed for each of the CRAS deletion genes. The ratio of the intensity of the specific mouse band to the endogenous hamster band was determined by densitometry and compared with expression of the intact H3.2 gene. Table 1 summarizes the results of this analysis. The H3h bands shown in Figure 2 are overexposed to allow visualization of the mouse-specific bands. The intensity of the H3h bands was quantitated on less exposed films. Although the reader may not be able to make all of the comparisons by eye, some of the differences can be easily seen. For example, if the H3m bands in lanes 1 and 9 of Figure 2 are directly compared, it is readily apparent that the total amount of probe protected by mouse mRNA molecules is comparable. However, the intensities of the bands in lanes 1 and 9, produced by protection of the probe by hamster H3.2 mRNA molecules, are quite different. This difference in the ratios obtained by comparison of the intensity of the mouse band with that of the endogenous hamster band in lanes 1 and 9 accounts for the 4-fold drop in expression reported in Table 1 for the H3.2 CRAS4 deletion gene.

Gene H3.2CRAS1a showed expression levels not significantly less than that of the intact parent H3 gene. H3.2CRAS1a and H3.2CRAS3 served as internal controls, showing that the CRAS region can be mutated, reassembled into an H3 gene and transfected into CHO cells and yield normal expression. However, this was not the result obtained with gene H3.2CRAS2. As is shown in Table 1, deletion of 24 nt (see Figure 1) resulted in 39% of the expression of the wild-type gene. Data are not shown for analysis of mutant gene H3.2 CRAS1b (see Fig. 1), but identical S1 nuclease protection analysis of RNA from stably

### Table 1. Effects of site-directed deletion or mutation in the H3.2 CRAS upon expression and DNA–protein interactions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative expression (%)</th>
<th>Ave</th>
<th>α Complexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3.2 (intact)</td>
<td>100 (2)</td>
<td>100 (2)</td>
<td>+</td>
</tr>
<tr>
<td>H3.2CRAS1a</td>
<td>77 (2)</td>
<td>91 (3)</td>
<td>+</td>
</tr>
<tr>
<td>H3.2CRAS2</td>
<td>37 (2)</td>
<td>43 (2)</td>
<td>+</td>
</tr>
<tr>
<td>H3.2CRAS3</td>
<td>88 (2)</td>
<td>100 (2)</td>
<td>+</td>
</tr>
<tr>
<td>H3.2CRAS4</td>
<td>24 (2)</td>
<td>33 (3)</td>
<td>28</td>
</tr>
<tr>
<td>H3.2CRAS</td>
<td>30 (4)</td>
<td>31 (3)</td>
<td>39</td>
</tr>
</tbody>
</table>

a The autoradiograph used in experiment 1 is shown in Figure 2 and, similarly, experiment 4 is shown in Figure 6B. S1 nuclease protection assays were used in experiments 1–5.

b These results were obtained by electrophoretic mobility shift analysis, shown in Figures 3, 4 and 6.

Results of experiment 4 were quantitated by comparison between c.p.m. in specific and endogenous bands by direct exposure of the gel (Betagen). Values given above in all columns are the averages of determinations using independent pools of transfected cells (number of pools used is in parentheses after each percentage). Values are calculated by comparison of the ratio obtained for the gene construct to the ratio determined for the transfected intact H3.2 gene, which is arbitrarily set at 100% expression.

d In experiment 6 expression was quantified by RNase protection assay utilizing a single RNA probe that maps the 3' region of the intact H3.2 mRNAs as well as the CRAS mutant mRNAs. In experiments 1, 2, 3 and 5 autoradiographs were quantitated by densitometry (PDI) of films exposed for the appropriate amount of time, for example, endogenous bands are overexposed on films used to quantitate the CRAS4 and CRAS α specific bands. The difference in exposure time was then included in the calculation of the ratio of the density of the specific band to that of the endogenous band in a given lane.
transfected cells showed a drop in expression similar to that observed in cells transfected with the CRAS2 deletion. The CRAS1b and CRAS2 deletions overlap, carrying deletions of an identical 12 nt. The difference in expression between the intact gene and the CRAS2 deletion gene is visible to the unassisted eye; direct comparison of the intensity of the mouse- and hamster-specific bands in lanes 1 (intact H3.2) and 5 (H3.2 CRAS2) shows that expression of the CRAS 2 deletion mutant is significantly decreased when assayed in pools of stable transfectants.

Expression of mutant H3.2CRAS3 was close to normal. Direct eye comparison of lanes 5, 7 and 9 verifies densitometric analyses, i.e. CRAS3 deletion has much less effect on expression than CRAS2 or CRAS4 deletion. For the mutant gene designated H3.2CRAS4 deletion of the 24 nt indicated in Figure 1 resulted in a 4-fold drop in expression, as stated above. Thus these studies identify two regions that play a role in regulation of expression of the H3.2 gene. Here we will concentrate on the region identified by the CRAS4 deletion. We will focus on the CRAS2 deletion in a separate paper.

**In vitro analysis of nuclear protein interactions with the mutant CRAS fragments**

Next we examined the effect of those deletions whose effects on expression were examined in Figure 2 on *in vitro* ability to interact with nuclear proteins. Two major complexes are detected in the electrophoretic mobility shift assay shown in Figure 3 with the H3 CRAS as probe. We designate these complexes α and α' (lane 1 of Fig. 3A). The amount of α complexes observed in each extract will be measured directly in Figures 4 and 5 that these complexes map to the same nucleotides in the H3.2 CRAS as do the α complexes. Figure 3A shows the results obtained with the H3CRAS1a, H3CRAS1b, H3CRAS2, H3CRAS3 and H3CRAS4 fragments, which are restriction fragments excised from the deletion genes transfected for the studies shown in Figure 2. Binding conditions are described in Materials and Methods. Under these conditions, utilizing the intact H3 CRAS fragment, ~25% of the labeled probe is shifted to complex α.

As is seen in Figure 3A, deletion mutations 1a, 1b, 2 and 3 have no effect on formation of the α or α' complexes (lanes 1–4), but the CRAS4 mutation completely eliminates formation of the α complex (lane 5), leading to the conclusion that the nucleotides involved in this DNA–protein interaction are located within the 24 bases deleted in the CRAS4 mutation (see Fig. 1). Note that in addition to loss of the α complex, the α' complex is missing from lane 5.

We have previously shown that intact H3.2 and H2a.2 CRAS fragments form similar specific DNA–protein complexes easily competed for by the other CRAS fragment (13). Figure 3B shows that the protein(s) involved in forming the α complexes with the H3 CRAS forms an identical complex with the H2a CRAS. In this competition experiment 100x unlabeled synthetic H3.2 CRAS α duplex (Materials and Methods) was used to compete for the radioactively labeled H3 or H2a CRAS restriction fragments (lanes 2 and 4). This competitor oligonucleotide duplex contains the 7 nt match between the H2a and H3 CRAS (Fig. 1). The specificity of the H3.2 CRAS α complexes is further verified in a later figure. These experiments will show that the α DNA–protein interaction is highly dependent on the nucleotide sequence of the target DNA.

**Localization of protein binding domains within the CRAS by ‘stairway’ assay**

The data presented in Figures 2 and 3 localized the protein–H3 CRAS interactions, designated α complexes, to nucleotides located between nt 250 and 273 of the H3 CRAS, the nucleotides deleted in the H3.2 CRAS4 mutation. To determine more accurately the sequences involved in formation of the α complexes we labeled the native H3.2 or H2a.2 CRAS fragments on one end and successively deleted fragments using restriction enzymes (35). The shortened fragments were then used as probes in mobility shift assays. Figure 4A shows gel mobility shift assays of the CRAS fragment labeled on the 3' end at the HindIII site in the vector. Lanes 2–4 show that removing nucleotides up to the ScaI site (nt 263 of the H3 CRAS in Fig. 1) does not interfere with formation of the α complexes. Removal of nucleotides up to the HaeIII site (nt 266) causes a decrease in the amount of complex observed, but a portion of the α sequence must be present in nt 266–282, because a small amount of α complex is formed. Digestion with HhaI, which cuts at nt 277, causes a complete loss of ability to form the α complex with mouse nuclear proteins. This result implies that the binding site involves several nucleotides located between 266 and 282 in Figure 1, consistent with results obtained with the deletion gene H3.2 CRAS4, in which nt 249–273 are deleted. This result localizes the binding site for the α complexes in or near to the 7 nt that form one of two matches of 7 nt between H2a and H3, nt 270–276.
Figure 4. Localization of protein binding domains within the CRAS. In both panels of the figure the restriction fragment incubated with nuclear extract is diagrammed beneath the figure. Restriction sites used are indicated on the drawing. The letter and number to the left of the probe drawings indicate the lane on the panel. The presence or absence of formation of the α DNA-protein complexes is indicated by + or −. (A) H3.2 CRAS Sall–PstI subclone (13) 3′-end-labeled at the HindIII site in the vector. (B) Lanes 1–4, H2a.2 CRAS AccI–PstI subclone (13) 3′-end-labeled at the EcoRI site in the vector; lanes 5–8, H2a.2 CRAS 3′-end-labeled at the HindIII site in the vector.

The H3 CRAS fragment was then labeled on the 5′ end (AvaI in the vector) and similarly analyzed as described above (data not shown). Restriction fragments produced with BgII, which cuts at nt 271 and removes only 11 nt of the H3.2 CRAS, causes a complete loss of α CRAS–protein complexes. This result confirms that the binding site for the α protein is located between nt 268 and 282, again implicating the conserved sequence located between nt 270 and 276.

The H2a.2 CRAS was then subjected to similar analysis by creation of successive deletions with restriction enzymes. Figure 4B shows the resulting ‘stairway’ analysis of the H2a CRAS fragment in EMSA. Figure 4B, lane 4 shows the intact H2a CRAS fragment 3′-end-labeled at a 5′ EcoRI site in the vector polylinker. Lanes 1, 2 and 3 show that removal of the 3′-end of the fragment (back to nt 172) does not interfere with formation of the α complex. Cutting back to nt 172 produces a fragment containing only 22 nt of the H2a.2 CRAS, yet it still forms α complexes with nuclear extract (lane 1). Not surprisingly, this fragment also contains the α sequence match with the H3.2 CRAS, located between nt 153 and 159 in the H2a CRAS (Fig. 1). Figure 4B, Lane 5 shows the intact H2a fragment 3′-end-labeled at the HindIII site in the vector. Figure 4B, lane 6 shows that removal
of the first 22 nt of the H2a.2 CRAS by RsaI digestion causes total loss of α complexes. This result confirms those observed in Figure 4B, lane 1, that the binding site for the α complexes is located between nt 150 and 172 in the H2a.2 CRAS. Figure 4B, lanes 7 and 8 show the expected result, that further loss of the 5' half of the H2a CRAS causes loss of the α binding site. The results obtained in this experiment implicate the conserved α sequence, nt 153–159, as the CRAS α binding site in the H2a CRAS.

Nucleotide–protein contacts within the CRAS α box

Figure 5 contains the results of DNase I footprint and methylation interference experiments that identify the bases within the CRAS α box that make contact with the protein that binds to this CRAS element. Figure 5A includes DNase I protection analysis of the labeled H3 CRAS fragment, end-labeled on the coding or non-coding strand as indicated. The DNase I-digested protein–DNA complexes were loaded onto a 4% native gel and free probe (F) separated from bound (B). The eluted products were analyzed on an 8% sequencing gel. Maxam–Gilbert A+G reactions were loaded in lane 1 (3'-end-labeled coding strand) and lane 10 (5'-end-labeled non-coding strand). Lanes 2, 3, 8 and 9 included reactions with 25 ng DNase I and lanes 4–7 reactions with 12.5 ng. As is clearly visible in the bound lanes at both concentrations of DNase I and on both strands, the conserved H3 CRAS α box sequence, CATGGCG, is protected from DNase I digestion by binding of a nuclear protein. The protection extends several bases 5' of the conserved sequence on the coding strand, so that at least 12 bases (GCCGTCATGGCG) are protected by the α box binding protein. Protection on the non-coding strand includes the consensus nucleotides and evidence for protein interaction extends to the G residue in the third position before the initial nucleotide (G) of the consensus sequence. This base shows an increase in intensity in the bound lanes (hypersensitive band), but no evidence for interaction beyond this base is observed on the non-coding strand. Clearly, protection on this strand does not extend as far as on the other strand.

Figure 5B and C shows the effects of G methylation reactions on protein interactions with the CRAS α sequence. If methylation of a G nucleotide interferes with protein interactions, the cleavage product at that position on the gel is enriched in the unbound (free) probe lane. As is clearly observed in reactions utilizing 5'-end-labeled probe and the non-coding strand (Fig. 5B, lanes 1 and 2), as well as 3'-end-labeled probe and the coding strand (Fig. 5C, lanes 1 and 2), the α box consensus G nucleotides show evidence of methylation interference. As observed in the DNase I protection analysis (Fig. 5A), the DNA–protein interaction defined by methylation interference also extends several nucleotides 5' of the initial base (C or its complement G) of the α consensus sequence. However, the contacts do not extend beyond the terminal G at the 3'-end of the α consensus nucleotides. Figure 5B, lanes 3 and 4 and Figure 5C, lanes 3 and 4 show the results of excising the rapidly migrating probe–protein complexes previously referred to as α' complexes (Fig. 3A). The α complexes (lanes 1 and 2) were also excised from a native gel and directly compared with the α' reaction cleavage products. These α' methylation products show identity with the α complexes in lanes 1 and 2 of Figure 5B (5' probe) and Figure 5C (3' probe). These results shows that the same DNA binding protein is involved in the formation of both complexes. Because the rapidly migrating complex is not
observed in all extracts and the amount of this complex increases upon repeated freezing and thawing of extracts, we believe that the α complexes are the result of proteolytic cleavage of the α box DNA binding activity.

**In vitro and in vivo effects of mutation of the 7 nt of the CRAS α box sequence confirm its role in histone gene expression**

In Figure 5A-C we showed that the 7 nt of the CRAS α box make direct contact with the nuclear proteins that form the α complex observed in mobility shift assays. In Figure 6A we show directly that mutation of the 7 bp CRAS α sequence, the H3 CRASmα gene, is sufficient to abolish formation of CRAS α complexes completely in EMSA. The mutant gene was produced by oligonucleotide-directed mutagenesis, changing the 7 nt α sequence CATGGCG to TTCTAGA. The mutant CRAS fragment was removed from the gene and end-labeled as previously described. As is clearly seen in Figure 6A, lane 2 this mutated CRAS is no longer capable of interacting with nuclear proteins to form the α complex. The probe used in lane 1 is the native H3.2 CRAS. Figure 6B shows the results of S1 analysis of expression of the gene carrying this 7 nt mutation. This figure shows that expression of the mutated gene is nearly identical to that of the deletion 4 gene. RNA was isolated from CHO cells stably transfected with the gene of interest and assayed with homologous probes. The reactions were as follows: lanes 1 and 7, intact H3.2; lanes 2–4 and 8, H3.2 CRASmα (7 nt mutation); lanes 5, 6 and 9, H3.2 CRAS4 (deletion gene, Fig. 1). H3h refers to probe fragments protected by hamster transcripts and H3m refers to fragments resulting from protection by mouse transcripts from the transfected genes. Independent pools of transfectants were the source of RNA for reactions in lanes 1–6. Lanes 7–9 are the result of combining three independent pools of transfectants in a composite assay, for direct comparison with composite reactions from pools of cells stably transfected with the other two genes. Comparisons of relative expression were done both by direct quantification (Betascope) and by densitometric analysis, as described for Figure 2. The ratio of the intensity of the hamster band to that of the mouse band was compared with that of the intact gene. The film shown here was overexposed for the hamster bands to show the mouse bands clearly. Shorter exposure times were used to quantify the hamster bands by densitometry. Results of these analyses are given in Table 1 and verify that the 4-fold reaction observed in the H3.2 CRAS4 transfectants is due to loss of the 7 nt CRAS α element. Direct mutation of the 7 nt of the CRAS α box causes a 4-fold drop in expression, as does deletion of 27 nt that include the α box nucleotides, the CRAS4 deletion (see Figs 1 and 2 and Table 1). Because the sequence of the CRAS α fragment is identical to the H3.2 sequence given in Figure 1, with the exception of the 7 nt, these nucleotides are necessary for the formation of the CRAS α complex.

**A replication-independent H3 gene contains a mutant α box element**

The ability of the comparable coding region sequence of a non-replication-dependent histone gene was examined for its ability to bind nuclear proteins and compete with the H3.2 α box for binding in EMSA competition experiments. The coding sequence of the H3.3 gene is 67% identical to the H3.2 gene, primarily as a result of third base changes (30–32), but the H3.3

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**Figure 6. Mutation of the CRAS α element abolishes formation of α complexes in vitro and causes a 4-fold drop in in vivo expression.** (A) EMSA of the end-labeled H3CRAS α fragment with mouse nuclear extract (Materials and Methods). Lane 1, native H3.2 CRAS fragment; lane 2, mutant CRAS fragment. The CRAS α fragment is identical to the wild-type H3.2 CRAS except for the 7 nt of the CRAS α box sequence, CATGGCG, which is mutated to TTCTAGA.Lane 3 shows the results obtained when the H3CRAS α box is mutated to TTCTAGA. (B) Expression of the CRAS α mutant gene in stably transfected CHO cells. S1 nuclease protection assay of total RNA from pools of stable transfectants is shown here. Homologous probes were utilized for each gene. Conditions were similar to those described in the legend to Figure 2. Results of quantitation are shown in Table 1 (experiment 4).

**Figure 7. Sequence comparison of replication-dependent H3.2 CRAS and a non-replication-dependent H3.3 gene.** Nucleotide number is indicated above the aligned sequences; nt 171 of the H3.2 sequence corresponds directly to the H3.2 CRAS nt 171 in Figure 1. The H3.2 CRAS sequence is underlined. Asterisks denote nucleotide changes in the H3.3 sequence. The nucleotides corresponding to the two 7 nt matches between H3.2 and H2a.2 are indicated by bold.

*‘CRAS’ sequence contains clusters of mutations and the ‘α’ box shows a 4-fold drop in expression. A partial comparison of the H3.2 and H3.3 coding-region nucleotide sequences is shown in Figure 7. The results of EMSA experiments utilizing the H3.3 ‘CRAS’ are shown in Figure 8. Figure 8A shows the results obtained when the 3′-end-labeled H3.2 CRAS was incubated with mouse nuclear extract (lane 1) and competed with 100× H3.2 CRAS α duplex oligonucleotides (lane 2) or 100× H3.3 ‘CRAS α’ duplex oligonucleotides (lane 3). The sequence of these synthetic molecules is shown in Materials and Methods. The nucleotide sequences of these two synthetic duplex molecules differ at 11 positions (see also Fig. 7). The H3.3 ‘CRAS α’ oligonucleotide...*
We have identified an element through expression in a H3.2 histone gene and in vitro DNA–protein interaction experiments that is involved in control of high expression of replication-dependent histone genes. In vivo expression analysis by nuclease protection assays (Fig. 2) has shown that deletion of 24 nt that include the CRAS α element causes a 4-fold drop in expression of the H3.2 gene after stable integration into the hamster cell genome. We showed by ‘stairway’ analysis, DNase I footprinting and methylation interference and then directly by mutagenesis that 7 nt in the H3.2 CRAS, one of the two 7 nt matches between the H3 and H2a CRAS, interact directly with nuclear protein(s) and produce the α DNA–protein complexes. As previously stated, this element (the H3.2 CRAS α box) is identical in the core 7 nt to a sequence in the H2a.2 CRAS. We showed in Figure 3B that the H3 CRAS α box oligonucleotides compete with the H2a CRAS α complex (previously shown utilising the two CRAS restriction fragments in 13). We have confirmed that these H2a sequences are responsible for the α complexes formed on the H2a CRAS fragment (Fig. 4C and unpublished results). We then directly mutated the H3 CRAS α sequence, CATGGCG, and mutation of these 7 nt in the H3.2 CRAS was sufficient to totally abolish formation of α complexes (Fig. 6A). In vivo analysis of the effect of mutation of the 7 nt of the CRAS α element upon expression in stably transduced CHO cells showed a 4-fold drop in expression (Fig. 6B). These results are similar to results presented in Figure 2 of deletion of 24 nt in this region of the CRAS (the CRAS4 gene). We have shown in this paper a direct correlation between the ability to form α complexes in vitro and loss of expression in vivo (Figs 2 and 6B). Our results conclusively show that the CRAS α element plays an important role in normal expression of a mouse H3 gene, as well as an H2a gene. The histone CRAS α element is the first example of a conserved, common element involved in regulation of more than one class of histone genes.

Experiments are underway to further define the sequences within the H3.2 CRAS which are required for high expression of the H3.2 gene, specifically those deleted in the H3.2 CRAS2 gene. This deletion gene has led to the identification of a second CRAS element required for correct expression of the mouse H3.2 gene, the CRAS α element (submitted for publication). Both positive and negative regulatory elements may be present in the CRAS and deletion mutation effects on expression in vivo may not be straightforward, i.e. may not cause additive losses in expression. Cooperativity in binding at these sites might also lead to results from specific mutations within the CRAS that are not additive. We will address these questions in the future.

A replication-independent H3 gene, the H3.3 variant, does not have the CRAS α box in its coding sequence

As stated in the Introduction, various laboratories have identified class-specific promoter elements implicated in cell cycle regulation of human and mouse histone genes (7–12). Common factors necessary for correct expression of histone genes of more than a single class (other than those common to genes transcribed by RNA polymerase II) have not been previously reported. The data presented here show that a common intragenic element, the CRAS α box, is involved in the regulation of at least two classes of histone genes, H2a and H3.

Although we have no direct evidence for the involvement of the CRAS α element in cell cycle regulation, the in vivo function of this element in the H3 CRAS in correct cell cycle regulation of replication-dependent histone genes is implicated by comparisons
with the protein binding abilities of the α box sequence from a replication-independent H3.3 gene to those of the H3.2 (replication-dependent) gene. Duplex oligonucleotides for the H3.2 and H3.3 α box elements were synthesized. The H3.3 oligonucleotides were unable to bind the nuclear factors bound by the α box found in the replication-dependent H3.2 and H2a2 genes. The amino acid sequences of the H3.2 and H3.3 gene products are very similar (both have third base substitutions throughout the H3.3 coding sequence; nucleotide homology 67%), but the α box sequence of H3.3 is much less similar at the nucleotide level than the rest of the coding sequence; the H3.3 α box shows 5 out of 7 nt mismatches. It is notable that the CRAS α box sequence is mutated in the H3.3 sequence to encode the characteristic amino acids unique to every vertebrate H3.3 protein known, specifically codons 89 and 90 (nt 268-273 in Fig. 7) (36-38). The absence of this protein binding element in the non-replication-dependent H3.3 genes supports our hypothesis that the CRAS is involved in correct regulation of the mouse H3.2 and H2a2 genes we have examined in the cell cycle. It is intriguing that the major mutations of the H3 amino acid sequence at codons 89 and 90 were described as diagnostic of variant H3.3 proteins several years ago (36-38). The high degree of conservation of histone encoding sequences within each class of histone from plants to man makes it attractive to speculate that the CRAS element is involved in expression of all replication-dependent histone genes. The potentially deleterious effects of mutations within the CRAS on cells unable to package newly replicated DNA properly is one explanation for the high degree of conservation long noted among histone genes of all classes.

Summary

In conclusion, these data show that the CRAS α box plays an important role in transcription of two highly expressed replication-dependent mouse histone genes and this element may mediate a common transcriptional control mechanism in all replication-dependent histone genes. The role of the CRAS α element in regulation of the other classes of histone genes is under investigation. Natural mutation of the CRAS α element in an H3 gene that is not coordinately regulated with DNA synthesis suggests that this element is required for correct regulation in the eukaryotic cell cycle.

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