Identification of a second conserved element within the coding sequence of a mouse H3 histone gene that interacts with nuclear factors and is necessary for normal expression

Nikola K. Kaludov, Lil Pabón-Peña and Myra M. Hurt*

Department of Biological Science, Florida State University, Tallahassee, FL 32306-3050, USA

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ABSTRACT

Replication-dependent histone genes of all four nucleosomal classes are coordinately up-regulated at the beginning of S phase of the eukaryotic cell cycle. The universality and importance of this process in eukaryotic cells suggest that common regulatory mechanisms are involved in controlling the high level of expression of these histone genes. We have previously identified the α element within mouse H2a.2 and H3.2 coding region activating sequences (CRAS), which is involved in regulation of these two replication-dependent genes. Here we report the identification of a second element within the mouse histone CRAS, the Ω element. This element interacts with nuclear proteins and we present in vivo evidence that this sequence is required for normal expression. Ω nucleotides involved in interaction with nuclear proteins have been precisely mapped by means of DNase I footprinting and methylation interference assays. A naturally occurring mutation in the Ω sequence is found in a replication-independent H3.3 gene. Mutation of the H3.2 Ω element to that of the H3.3 sequence (3 nt changes) caused a 4-fold drop in in vivo expression of the H3.2 gene in stably transfected CHO cells, equaling the effect of mutation of all 7 nt of the element. By UV cross-linking we have determined the approximate molecular weight of the Ω binding protein to be 45 kDa. Finally, we identify putative Ω sequences in the coding region of mouse H2b and H4 histone genes.

INTRODUCTION

Histone genes are classified as either replication-dependent or replication-independent (1). Expression of the former is coordinately up-regulated at the onset of DNA synthesis in the eukaryotic cell cycle, supporting the cellular requirement for histone proteins for packaging the newly replicated DNA into nucleosomes and higher order chromatin structures (2–4). Cell cycle-dependent histone gene expression is coordinately regulated at both the transcriptional and post-transcriptional levels, but the exact molecular mechanisms of this regulation in higher eukaryotes are unknown (5,6). In contrast, expression of the replication-independent genes is low and constitutive throughout the cell cycle. These genes encode the so-called replacement variant histones. Their products accumulate to higher levels only in non-dividing cells (7–10).

In vivo and in vitro studies have demonstrated that increased transcription of histone genes during S phase is in part regulated through promoter-proximal DNA sequences and their cognate transcription factors (5,6,11–19), but these sequences are class specific and are not shared among the different histone classes (12–14). We previously identified coding region activating sequences (CRAS) required for high expression of at least two (H3.2 and H2a.2) replication-dependent mouse histone genes (20,21). Deletion of either CRAS caused a 20-fold drop in expression of H3.2 and H2a.2. In addition, using stable transfections of mouse histone gene constructs into CHO cells, we showed that the H3.2 CRAS could restore high expression to a H2a.2 gene with the CRAS deleted (20). Subsequently we identified an element within the CRAS, the α element, that interacts specifically with nuclear proteins and is required for normal expression of the H3.2 gene in vivo (22). We showed that the H3.2 and H2a.2 α element–protein interactions are identical (22).

Here we report the involvement of a second conserved element, the CRAS Ω element, which we previously identified as one of two 7 nt matches between the H2a.2 and H3.2 CRAS (22). We present here several lines of evidence, both in vivo and in vitro, that demonstrate the importance of the sequence to normal histone gene expression. (i) The Ω element is the target for nuclear protein interactions; electrophoretic mobility shift assays (EMSA), including cross-competition experiments, show that these DNA–protein interactions are similar (if not identical) for all four classes of histone genes used in this study. DNase I footprinting and methylation interference analyses have identified the precise nucleotides involved in the H3.2 DNA–protein interactions. By UV cross-linking we have determined the approximate molecular weight of the Ω binding protein (~45 kDa). (ii) Using nuclease protection analysis to quantify specific

* To whom correspondence should be addressed
Mouse myeloma cells were grown in spinner cultures to a density of 5 × 10^5 cells/ml in Dulbecco’s modified Eagle’s medium, 10% horse serum and 5% CO_2 at 37°C. Nuclear extracts were prepared by our modification of the method of Shapiro et al. (24).

**MATERIALS AND METHODS**

**Nuclear extracts**

Mouse myeloma cells were grown in spinner cultures to a density of 5 × 10^5 cells/ml in Dulbecco’s modified Eagle’s medium, 10% horse serum and 5% CO_2 at 37°C. Nuclear extracts were prepared by our (20) modification of the method of Shapiro et al. (24).

**Partial purification of Ω binding factors**

Nuclear extracts were applied to DNA–cellulose resin (40 ml bed volume; Pharmacia) equilibrated with buffer (20 mM HEPES, pH 7.9, 0.2 mM EGTA, 0.2 mM EDTA, 100 mM KCl, 20% glycerol and 2 mM dithiothreitol). Proteins were eluted with the same buffer containing 200 or 400 mM or 1 M KCl. Fractions were analyzed for CRAS binding activity by EMSA. The fractions containing the CRAS binding activity were applied to a DEAE–Sephareose resin (40 ml bed volume; Pharmacia) equilibrated in the same buffer and the proteins eluted in the same manner as from the DNA–cellulose column. The fractions that contained the CRAS Ω binding activity (0.2 M fractions) were pooled and used in subsequent experiments.

**Electrophoretic mobility shift assay (EMSA)**

The mouse H3.2-614 SauIII–PstI, H2a.2-614 Accl–PstI and H4–12 SacII–HaeIII restriction fragments were subcloned for use in EMSA (20,22,25). The H2b–143 NeoI–Alul restriction fragment was obtained directly from the cloned gene (GenBank accession no. X16148) into pGEM3Zf(+) (24). Oligonucleotide-directed mutagenesis was performed by the Kunkel method (30).

**Oligonucleotide-directed site-specific mutagenesis**

A SauIII–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 (GenBank, EMBL accession no. X16148) into pGEM3Zf(+). Oligonucleotide-directed mutagenesis was performed by the Kunkel method (30).

**Cell culture and stable transfections**

CHO cells were grown in McCoy’s 5A medium supplemented with 10% calf serum, 5% CO_2 at 37°C. Pools of stable transfectants were selected after co-transfection of the histone gene of interest with the pSVneo plasmid with the oncogenic polybrene and dimethylsulfoxide (31). Selection with the drug G418 started 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 the mouse histone genes (32).

**RNA isolation and analysis**

Cells were harvested at 50% confluence by trypsination and total RNA was prepared with a Biotec Laboratories Ultraspec RNA isolation system (33). The amount of histone mRNA for the gene of interest was quantified by nuclease protection assay. For S1 assays (34) specific 3’-end-labeled probes for each of the mRNA levels from stable transfectant CHO cells we show that mutation of the 7 nt of the H3.2 CRAS Ω element causes a 4-fold drop in expression. Moreover, similar analysis of the levels of mutant mRNAs in cells stably transfected with a double mutant gene, containing altered CRAS α and Ω sequences, showed a >10-fold effect of mutation of both elements. This result indicates that in vivo cooperative interaction between these two elements in maintaining the high level of expression of the replication-dependent histone gene is likely. (iii) A mouse replication-independent histone gene, H3.3, contains a naturally mutated Ω element (3 out of 7 nt changed) which fails to interact with nuclear proteins directly or to compete for binding with the native H3.2 Ω element. Results obtained in vivo confirm that mutations changing the H3.2 Ω element to the H3.3 sequence (3 out of 7 nt changed) cause a drop in expression equal to that seen upon mutation of all 7 nt of the Ω sequence.

We have shown elsewhere that Ω binding activity is present only in G1 phase nuclear extracts (23). The results presented herein provide additional evidence that the CRAS Ω element plays an important role in regulation of expression of replication-dependent histone genes in the cell cycle.
CRAS mutants, as well as the intact gene, were produced. Each mutant plasmid was linearized at the NcoI restriction site, which spans the initiation codon of the H3.2 gene. The ends were filled with Klenow fragment using αspans the initiation codon of the H3.2 gene. The ends were filled mutant plasmid was linearized at the CRAS mutants, as well as the intact gene, were produced. Each ranged between 5 × 105 and 1 × 106 c.p.m./µg (22). S1 nuclease-protected fragments were separated on 6% polyacrylamide–8 M urea gels. Gels were dried and either directly quantified with a Betascope and/or autoradiographed for densitometric analysis. Relative expression was determined by calculation of the ratio of the endogenous hamster H3 band to the specific mouse H3 band. The ratio obtained for the intact mouse H3.2 gene was arbitrarily set as 100% and the ratio obtained for the mutant genes was then compared with the wild-type. Alternative-

**DNase I footprint analysis**

Binding reactions (50 µl) contained 7.5 ng labeled CRAS fragment, 10 µg poly(dl–dc) (Pharmacia) and 30 µg protein (partially purified nuclear extract). DNase I (5 or 10 ng; Pharmacia) digestions were performed as described by Singh et al. (36). Free and bound probe molecules were separated on a 6% native gel eluted at 37°C overnight and analyzed on an 8% polyacrylamide–8 M urea sequencing gel. Radioactivity was quantified by autoradiography. Films were scanned with a PDI (Protein & DNA Imageware Systems) film scanner.

**Methylation interference**

Labeled CRAS fragments were partially methylated at G (in the major groove) and A (in the minor groove) residues with dimethyl sulfate (DMS) as described by Siebenlist and Gilbert (37). Labeled probe molecules (0.2 pmol) were methylated with 1 µl DMS for 1 min at room temperature in 100 µl total volume and precipitated. Binding reactions were as described for DNase I footprinting except that they contained 7.5 ng modified probe. Free and bound molecules were separated as described above, excised from the gel and electroeluted from the gel slice. They were then cleaved with 100 µl 1 M piperidine at 90°C for 15 min (38). The cleavage products were analyzed on an 8% sequencing gel. Autoradiographs were quantified as above.

**UV cross-linking**

Oligonucleotides used in crosslinking experiments were synthesized for the H3.2 Ω element:

H3.2 (1) 5′–CTTGCGCGATCTGGCGACCGAGCCG (noncoding)  
H3.2 (2) AGAGCGGTGTTCGCG–5′ (coding)

The short oligonucleotide was annealed to the full-length complementary oligonucleotide strand by mixing at the same molar ratio followed by incubations at 88°C for 2 min, 65°C for 10 min, 37°C for 10 min, 25°C for 5 min and 4°C for 5 min. The oligonucleotide duplex was incubated with 5-bromo-2′-deoxyuridine 5′-triphosphate (0.2 mM final concentration), [α-32P]dCTP and the Klenow fragment of DNA polymerase I for 5 min at room temperature. Then a mixture of dATP, dGTP and dTTP (0.4 mM final concentration) was added and the reaction was allowed to continue for 10 min at room temperature. The labeled oligonucleotides were then ethanol precipitated.

In cross-linking experiments the irradiation step on DNA–protein complexes was performed in one of two ways, in the gel (39) or in solution (40,41). For the former the labeled oligonucleotide duplex (7.5 ng), poly(dl–dc) (0.2 µg/µl final concentration; Pharmacia) and 30 µg crude mouse myeloma nuclear extract were incubated in binding buffer (50 µl total reaction volume; see above) for 20 min at 4°C, loaded on a 6% native
polyacrylamide gel and separated by electrophoresis at 200 V. The Ω box complex was visualized by autoradiography and excised from the gel. The gel slice was irradiated for 20 min at room temperature with an inverted UV light (300 nm peak, gel slice 5 cm from the light source). Proteins were electroeluted from the gel slices and the samples dried and resuspended in loading buffer (62.5 mM Tris–HCl, pH 8.0, 0.5 M glycin, 0.25% SDS). Proteins were then separated by electrophoresis on a 10% SDS–polyacrylamide gel and radioactive bands visualized by autoradiography after drying of the gel.

For experiments using UV cross-linking in solution the protocol was identical until the end of the binding reaction. The binding reaction was then directly irradiated with UV light as above. After irradiation the reactions were incubated with DNase I (5 µg) for 30 min at 4 °C. The samples were then dried, resuspended in loading buffer and loaded on a 10% SDS–polyacrylamide gel for electrophoretic separation at 200 V. The radioactive band was visualized as above.

RESULTS

Localization of the Ω element within the CRAS by ‘stairway’ assay

We have previously localized the H3.2 and H2a.2 CRAS between nt 171 and 282 (codons 58–93) and nt 147 and 255 (codons 50–85) respectively in the histone protein-encoding sequences (20). Deletion of the CRAS in the H3.2 and H2a.2 replication-dependent genes in stable transfectants caused a 20-fold drop in expression from that of the intact genes (21). In addition, the intact CRAS fragments in both genes were shown to compete for binding of similar, if not identical, mouse nuclear proteins (20,21).

A direct comparison between the H3.2 and H2a.2 CRAS sequences showed only two regions of 100% identity, the α and Ω sequences (22). The CRAS α element has been shown to interact specifically with nuclear protein(s) and is required for normal expression (22). However, mutating or deleting the α sequence accounted for a 4-fold drop in expression in vivo, whereas deletion of the full-length CRAS yields a 20-fold decrease. Therefore, other subsequences within the CRAS must also be involved in the high level of expression of replication-dependent histone genes. To determine whether the second homologous sequence was in fact involved in interactions with nuclear proteins we created a sequential set of deletions within the H3.2 CRAS using restriction enzymes. We had previously observed that deletion or mutation of the α binding site enhanced detection of the Ω binding activity. Conversely, the H3.3 ‘CRAS’ element within the CRAS by

Similar stairway analysis was performed with 3′-end-labeled fragments (data not shown), which also confirms that sequences within nt 185–226 are required for formation of the Ω complex. The data shown in this figure identity the conserved sequence found in the H3.2 and H2a.2 CRAS, -CGAGA TC-, located at nt 219–225 in the H3.2 CRAS, as the binding site for the CRAS Ω complex.

Specificity of the CRAS Ω complex

Competition experiments showing specificity of the Ω element interactions with nuclear proteins utilized a naturally occurring mutant H3 gene, the mouse H3.3 gene, which is a replication-independent histone variant. The complete H3.2 CRAS sequence and the comparable H3.3 ‘CRAS’ sequence (from the replication-independent variant) are shown in Figure 2. Note that H3.2 and H3.3 are very similar, in fact, they are 67% identical at the nucleotide level and the differences are primarily due to third base changes (bold type) (27–29). However, the H3.3 ‘CRAS’ contains clusters of mutations. The H3.3 ‘α’ sequence has changes in 5 of 7 nt (in vivo and in vitro analysis of the CRAS α element is described elsewhere; 16). The H3.3 ‘Ω’ sequence differs from the H3.2 sequence at 3 of 7 nt.

An experiment comparing the effectiveness of the H3.2 and H3.3 Ω sequences as competitors is shown in Figure 3. In lanes 2 and 3 the H3.2 Ω duplex oligonucleotides are used as unlabeled competitors and efficiently compete for nuclear proteins with the labeled H3.2 CRAS fragment; a 50-fold molar excess eliminates Ω binding activity. Conversely, the H3.3 Ω duplex oligonucleotides showed no ability to compete with the H3.2 fragment for Ω complex formation, even in 100-fold excess (lanes 4 and 5). Comparison of the oligonucleotide duplexes (see legend to Fig. 3) shows that the H3.2 and the H3.3 competitor duplexes differ in only 4 of 22 nt. Results shown in this figure identity the H3.2 Ω sequence as the target for specific interactions with nuclear proteins, so specific that molecules 82% identical have little if any effect as competitors even when present in 100-fold excess.
**Figure 3.** High degree of specificity of CRAS Ω element–protein interactions. Sequences of the competitor oligonucleotides were as follows:

H3.2 CRAS Ω box duplex,
GTGCGCGAGATCGCGCAGGACT
CGCTCTAGCGCGTCCTGAcacg;

H3.3 CRAS Ω box duplex,
GTGCGAGAAATTGCTCAGGACT
CTCTTTAACGAGTCCTGAcacg.

The H3.3 molecules differ from the H3.2 duplex at only four positions out of 22 nt on each strand. Lane 1 contains the complete binding reaction minus specific competitor; lanes 2 and 3 contain a 50- or 100-fold excess of cold H3.2 duplex oligonucleotides and lanes 4 and 5 contain a 50- or 100-fold excess of cold H3.3 oligonucleotides. The probe was the mutant CRAS fragment H3.2αXba.

**Nucleotide–protein contacts in the CRAS Ω element**

Figure 4 shows the results of DNase I footprinting analysis of DNA–protein interactions involving the nucleotides of the H3.2 CRAS Ω element. Lanes 1 and 10 contain the Maxam–Gilbert G reaction (38) with these probes. Lanes 2, 3, 6 and 7 include reactions with 10 ng DNase I and lanes 4, 5, 8 and 9 reactions with 5 ng. Similar patterns of protection are seen with the two DNase concentrations. Both strands show evidence of strong interactions with proteins involving the 7 nt Ω consensus sequence and extending several bases beyond on both strands. Three hypersensitive sites (white squares on the figure) map the boundaries of these interactions on the coding strand at the higher DNase concentration. Interactions of the Ω protein(s) with the CRAS were quantified by densitometry and the ratio of the free to bound bands on the autoradiograph was determined for each individual band (presented graphically beneath the figure). The graphs show that certain bases are protected to a higher degree than others by the interacting protein(s), thus limiting DNase I access to the DNA. The higher the ratio of free to bound (higher bars on the graph) the stronger the contact between the protein and the base. This analysis shows definitively that the CRASΩ sequence is the binding site for the Ω complex observed in EMSA (Figs 1 and 3).

Methylation interference analysis of Ω–protein interactions confirmed the results of DNA footprinting analyses, as shown in Figure 5. If a methylated G (or A) nucleotide interfered with protein interaction, the cleavage product at that position would be missing (the band would decrease in intensity) in the bound lane and the free lane would be enriched. Densitometric analyses of the free:bound ratio are presented graphically at the bottom of the figure. The pattern of interference shown in this figure correlates exactly with that of the DNase I protection analysis shown in Figure 4.

Methylation of all G or A residues within the protected sequence shown in Figure 4 interfered with binding of nuclear protein(s), proving that the Ω sequence is the target for nuclear protein interactions observed in EMSA as CRAS Ω complexes. A summary of both sets of data is shown in Figure 6.

**The Ω sequence is required for normal expression of the H3.2 gene**

The relative expression of each of the H3.2 CRAS mutant genes stably transfected into CHO cells was determined by S1 nuclease protection assay (data not shown) and RNase protection assay.
The results of an RNase protection assay are shown in Figure 7. The RNA probe used in these experiments maps the 3′-end of the mouse (H3M) and endogenous hamster (H3H) transcripts, as shown in the diagram beneath the figure. At least three pools of stable transfectants were analyzed for each of the mutants (Table 1). Results of these experiments were quantified directly by comparison of the radioactivity (c.p.m.) in specific and endogenous bands (see Table 1). Relative expression of mutant genes was calculated by comparison with that of the transfected intact H3.2 gene, arbitrarily set as 100%. The summary of analyses of these genes is shown in Table 1. Mutation of all 7 nt of the H3.2 Ω element (H3.2ΩXho) resulted in a 4-fold drop in in vivo expression (Fig. 7, lane 3). The same effect was observed upon mutation of the H3.2 Ω sequence into that of the H3.3 (replication-independent) gene (3 of 7 nt changes) (Fig. 7, lane 6). These results show definitively that the Ω element plays an important role in regulation of expression of the replication-dependent H3.2 gene. Finally, mutation of both α and Ω elements resulted in a greater drop in expression (10- to 20-fold) than mutation of either the α or Ω sequences independently (Fig. 7, lanes 4 and 7).

UV cross-linking identifies a mouse nuclear factor that interacts with the H3.2 CRAS Ω element

Using the technique of UV cross-linking we have identified a mouse nuclear factor that interacts in a highly specific manner with the core nucleotides of the CRAS Ω sequence. Figure 8 represents the results of UV cross-linking experiments in gel (panel A) and in solution (panel B). With the oligonucleotides shown in Materials and Methods, an Ω binding site was synthesized that featured a bromodeoxyuridine (BrdU) residue in place of a thymidine on the coding strand (the T in the consensus CGAGATC). Upon activation (UV light irradiation) the BrdU residue covalently linked the bound protein to the radioactively labeled Ω binding site. After separation on a 10% SDS gel the molecular weight of the cross-linked protein could be approximated (39).

The results of the cross-linking experiments performed in the gel are shown in Figure 8A (lane 1, the UV-irradiated sample; lane 2, the non-irradiated sample). A radioactive band resulting from the nuclear protein covalently linked to the radioactive Ω oligonucleotides is observed in lane 1 and has an apparent molecular weight between 35 and 53 kDa. This band is not observed in the control lane.
Figure 7. The CRAS Ω sequence is required for normal expression of the H3.2 gene in vivo. The intact H3.2 and H3.2 CRAS mutant genes were stably co-transfected into CHO cells with neomycin resistance as the selective marker. Resistant cells were grown in independent pools for isolation of RNA. A single RNA probe was used to quantify all H3 transcripts, mouse (intact and mutant, denoted on the figure as H3αM) and endogenous hamster (H3α). The diagram beneath the figure shows the H3.2 gene; the asterisk denotes the restriction site (StuI) which was used to linearize the plasmid containing the H3.2 gene, pGEM3Zf(+). SP6 polymerase was used to synthesize a rUTP-labeled probe (600 nt in length, ending at the digestion site) using the SP6 promoter in the vector adjacent to the 3′ flanking sequence of the gene, which was then gel purified. The protection assay is described in Materials and Methods. Size of protected fragments is shown on the probe diagram. Lane 1, intact H3.2 (α, CATGGCG; Ω, CGAGATC); lane 2, H3.2CRASαXba (‘α’, TCTAGA; Ω, CGAGATC); lane 3, H3.2CRASCAXba (α, CATGGCG; Ω, CTCAGA); lane 4, H3.2CRASαXholΩXba (‘α’, TCTAGA; Ω, CTCAGA); lane 5, H3.2CRASαXholΩH3.3 (α, TGTTGCT; Ω, CGAGATC); lane 6, H3.2CRASΩH3.3 (α, CATGGCG; Ω, AGAAATT); lane 7, H3.2CRASαΩH3.3 (α, TGTTGCT; Ω, AGAAATT). Molecular weight markers are shown on the left of the figure.

Table 1. Effects of mutation within the H3.2 α and Ω sequences upon gene expression in stably transfected CHO cells.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative expression in experiment (%)</th>
<th>Average</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>H3.2, intact</td>
<td>100 (3)</td>
<td>100 (3)</td>
</tr>
<tr>
<td>H3.2ΩXho</td>
<td>25 (3)</td>
<td>25 (3)</td>
</tr>
<tr>
<td>H3.2ΩH3.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H3.2αXba</td>
<td>32 (4)</td>
<td>22 (3)</td>
</tr>
<tr>
<td>H3.2αΩH3.3</td>
<td>25 (3)</td>
<td>25 (3)</td>
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<tr>
<td>H3.2αΩXholΩXho</td>
<td>–</td>
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<td>H3.2αΩH3.3</td>
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Data for experiments 1–3 were obtained from S1 nuclease protection assays. Results from these experiments were quantified directly by comparison between c.p.m. in specific and endogenous bands by direct exposure of the gel (Betagen). Values are the averages of determinations using independent pools of transfected cells (number of pools used in parentheses). Values were calculated by comparison of the ratio obtained for the mutant gene with the ratio determined for the transfected intact H3.2 gene, which is arbitrarily set at 100% expression. In experiment 4 expression was quantified by RNase protection assay using a single RNA probe that maps the 3′ region of the intact H3.2 mRNAs and also the CRAS mutant mRNAs. Nucleotides mutated in these genes are described in the mutagenesis section of Materials and Methods.

This result was confirmed by a second type of experiment in which cross-linking occurred in solution (Materials and Methods). The results are shown in Figure 8B (lanes 1 and 2, control reactions as indicated on the figure; lane 3, the complete binding reaction minus UV irradiation; lane 4, the complete UV-irradiated Ω box binding reaction as in lane 3, but without cold H3.2 competitor. The molecular weight protein markers are drawn in lane 5.}

Figure 8. Identification of a nuclear factor that interacts specifically with the CRAS Ω element by UV cross-linking. UV cross-linking oligonucleotides used in these experiments are described in Materials and Methods. (A) In this experiment the protein–probe complexes were separated under our normal EMSA conditions prior to UV irradiation. Lane 1 represents the UV-irradiated box binding reaction. Lane 2 shows the same binding reaction without UV irradiation (control). The molecular weight protein markers are drawn in lane 3. (B) UV irradiation was performed in solution, as described in Materials and Methods. Lane 1, the Ω box binding reaction minus nuclear extract; lane 2, the complete binding reaction minus UV irradiation; lane 3, 100-fold excess of cold H3.2 Ω box duplex oligonucleotide added to the binding reaction; lane 4, the complete UV-irradiated Ω box binding reaction; lane 5, the complete UV-irradiated Ω box binding reaction minus UV irradiation (control). The molecular weight protein markers are drawn in lane 3.

This result was confirmed by a second type of experiment in which cross-linking occurred in solution (Materials and Methods). The results are shown in Figure 8B (lanes 1 and 2, control reactions as indicated on the figure; lane 3, the complete binding reaction minus UV irradiation; lane 4, the complete UV-irradiated Ω box binding reaction as in lane 3, but without cold H3.2 competitor. The molecular weight protein markers are drawn in lane 5. Other proteins are probably also involved in the low mobility CRAS Ω complexes observed in EMSA (data not available for this experiment).
The fact that mutating the H3.2 mutant form found in a replication-independent H3.3 gene (22) binds nuclear protein(s) is abolished in a naturally occurring transfectants, duplicating the effect of mutating all 7 nt of the 3 nt in the H3.2 gene caused a 4-fold drop in expression in stable found in a replication-independent H3.3 gene. This mutation of this sequence.

Restriction fragments from the coding regions of mouse H3.2, H2a.2, H2b-143 and H4-12 genes containing sequences highly related to the H3.2 CRAS Ω element were radioactively labeled and used in EMSA competition experiments (Fig. 9A). Lanes 1–3 show reactions using the H3.2 CRAS Ω fragment as probe: lanes 4–6, H2a; lanes 7–9, the H2b fragment; lanes 10–12, the H4 fragment. The DNA–Ω protein complexes observed in lanes 1, 4, 7 and 10 are very similar to each other, all migrating as low mobility complexes. Excess unlabeled H3.2 Ω oligonucleotides (50- and 100-fold molar excess) compete very effectively with the labeled fragments of all four classes for binding to nuclear proteins, as indicated on the figure. Figure 9B shows sequence comparisons between the H3 and H2a CRAS and the putative H2b and H4 Ω sequences from the H2b and H4 coding region fragments used in Figure 9A. The H3.2 and H2a.2 sequences are identical, whereas the putative Ω elements from both H2b-143 and H4-12 differ from the core heptad by one base (marked with an asterisk). The putative Ω elements in these four genes are in the same orientation within the coding sequence with respect to the transcription initiation site.

DISCUSSION

We have shown by stairway analysis and competition experiments that the H3.2 CRAS Ω sequence is a target for specific interaction with nuclear protein(s), which results in formation of the Ω complex (Figs 1 and 3). The high degree of specificity of this interaction is shown in Figure 3 in a competition experiment using sequences from a replication-independent H3.3 gene. As stated previously, the coding sequence of the H3.3 replacement variant histone gene is 67% identical to the H3.2 replication-dependent gene (Fig. 2; refs 27–29). The sequence differences are primarily due to third base changes. The H3.3 Ω′ sequence contains three changes in 7 nt and fails to bind nuclear factors (22) or to compete for binding (Fig. 3). We have used DNase I footprinting and methylation interference assays to confirm that the second 7 nt match (CRAS α being the first) between the H2α and H3 CRAS was involved in the CRAS Ω complexes (Figs 4–6). Further, we have shown that direct mutation of the conserved 7 nt of the Ω sequence caused a 4-fold drop in in vivo expression in stably transfected CHO cells, demonstrating a direct correlation between the ability of the Ω element to bind nuclear proteins in vitro and the loss of expression in vivo upon mutation of this sequence.

Subsequently we mutated the H3.2 Ω element in the replication-dependent gene, changing it to the comparable sequence found in a replication-independent H3.3 gene. This mutation of 3 nt in the H3.2 gene caused a 4-fold drop in expression in stable transfectants, duplicating the effect of mutating all 7 nt of the conserved Ω sequence (Fig. 7). The ability of this sequence to bind nuclear protein(s) is abolished in a naturally occurring mutant form found in a replication-independent H3.3 gene (22). The fact that mutating the H3.2 Ω sequence to that of the H3.3 gene causes a 4-fold drop in normal expression provides definitive evidence that the CRAS Ω element plays a key role in regulation of replication-dependent histone gene expression.

Figure 9. Four classes of histone genes contain sequences in the coding region that interact with the CRAS Ω proteins. Fragments from the coding sequence of the H2a, H2b and H4 genes, similar to the H3.2 CRAS, were end-labeled and used in electrophoretic mobility shift assays with partially purified Ω binding nuclear protein(s). (A) The results of cross competition experiments utilizing the H3.2 (lanes 1–3), H2a.2 (lanes 4–6), H2b-143 (lanes 7–9) and H4–12 (lanes 10–12) fragments as probes and unlabeled H3.2 Ω box duplex oligonucleotides in 50- (lanes 2, 5, 8 and 11) and 100-fold excess (lanes 3, 6, 9 and 12). Lanes 1, 4, 7 and 10 are normal reactions with the respective fragment and partially purified nuclear extract. (B) Sequence comparison between the H3, H2a and putative H2b and H4 Ω sequences. The sequences are aligned to show the homology between the four classes of histone genes. Nucleotide numbers represent nucleotide position with respect to the ATG codon. The sequence of all four Ω sequences is in bold. Differences from the core heptad are denoted by asterisks.

Sequences highly related to that of the H3.2 Ω element are found in the coding region of four classes of replication-dependent histone genes (Fig. 8B). Competition experiments using the H3.2 Ω oligonucleotides as competitors with labeled fragments from four classes of replication-dependent histone genes shows that identical DNA–protein complexes are formed in all four classes (Fig. 5A).

As mentioned before, the H3.2 and H2a.2 CRAS show 100% homology in two regions, the α and Ω elements (22,42). Mutation of the H3.2 CRAS α element alone in the H3.2 gene causes a
4-fold drop when expressed in stably transfected CHO cells. A similar drop in expression of H3.2 is observed when the Ω element is mutated, but a much greater decrease in expression of the gene is observed when both elements are mutated (10- to 20-fold), approaching the result obtained upon deletion of the entire CRAS (21). Cooperativity in binding at these sites is probably responsible for this result.

We have shown evidence here and elsewhere (22,23) to support our hypothesis that sequences located in the coding region of perhaps all replication-dependent histone genes are critical for normal high level expression. In addition, we have reported (23) that the CRAS α and Ω binding activities are present only in the G1 phase of the cell cycle. Our data shown herein (Figs 3 and 7) and elsewhere (22) demonstrate that naturally occurring mutations in the α and Ω sequences of a replication-independent H3.3 gene inactivate the in vivo and in vitro regulatory functions of these elements. These results provide strong support for our hypothesis that the CRAS elements play a major role in coordinating the expression of histone genes with the central regulatory processes of cell cycle progression in the mammalian cell.

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