Reduced histone biotinylation in multiple carboxylase deficiency patients: a nuclear role for holocarboxylase synthetase

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The attachment of biotin to apocarboxylases is catalyzed by holocarboxylase synthetase (HCS). An inherited deficiency of HCS results in the disorder ‘multiple carboxylase deficiency’, which is characterized by reduced activity of all biotin-dependent carboxylases. Here we show that the majority of HCS localizes to the nucleus rather than the cytoplasm based on immunofluorescence studies with antibodies to peptides and full length HCS and on the expression of recombinant HCS. Subnuclear fractionations indicate that HCS is associated with chromatin and the nuclear lamina, the latter in a discontinuous distribution in high salt-extracted nuclear membranes. During mitosis, HCS resolves into ring-like particles which co-localize with lamin B. Nuclear HCS retains its biotinylating activity and was shown to biotinylate purified histones in vitro. Significantly, fibroblasts from patients with HCS deficiency are severely deficient in histone biotinylation in addition to the deficiency of carboxylase activities. We propose that the role of HCS in histone modification may be linked to the participation of biotin in the regulation of gene expression or cell division and that affected patients may have additional disease beyond that due to the effect on carboxylases.

INTRODUCTION

Biotin is a water-soluble vitamin that is required for normal cellular metabolism and growth (1). It functions as a carboxyl carrier in carboxylation, decarboxylation and transcarboxylation reactions (2,3). In mammals, it is the cofactor of four different biotin-dependent carboxylases. Three are mitochondrial, propionyl-CoA carboxylase (PCC), pyruvate carboxylase and α-methylcrotonyl-CoA carboxylase, while the fourth, acetyl-CoA carboxylase, is present as two genetically distinct proteins, one in mitochondria (ACC2) and the other in the cytosol (ACC1). In each case, biotin is covalently bound to a lysine residue in a highly conserved domain common to all biotin-dependent carboxylases. Biotin attachment is catalyzed by a single enzyme, holocarboxylase synthetase, HCS (gene symbol, HLCS), in a two-step, ATP-dependent reaction in which biotin is first activated to biotinyl-5'-AMP and then transferred to the apocarboxylase substrate with release of AMP (3).

Biotin is synthesized in microorganisms, yeast and plants (4) and is secondarily available as a dietary nutrient in meat and other animal products. The description of children with an inherited deficiency of several carboxylase activities led to the discovery of HCS as the enzyme responsible for biotin attachment. HCS deficiency, as early-onset biotin-responsive multiple carboxylase deficiency (MCD), is an autosomal recessive disorder typified by infantile neurological, developmental and metabolic abnormalities, the latter including lactic acidosis, ketosis and organic aciduria that reflect impairment of the individual carboxylases due to mutations in the HLCS gene (1). The disease may be fatal, although most patients show a remarkable resolution of their symptoms by the administration of pharmacological doses of oral biotin. This is partially explained by the occurrence of most HLCS mutations in the biotin transfer domain of the enzyme which have been shown to result in a reduced affinity of the enzyme for biotin (5–7). A related disorder, generally of later onset, is biotinidase deficiency. It is also associated with deficient activity of all four carboxylases, but its cause is a block in the recycling of biotin from degraded protein (1,8). Biotinidase catalyzes the hydrolysis of biocytin (biotinyllysine) or small biotin containing peptide fragments (9,10).

While biotin has traditionally been viewed as a cofactor of carboxylases, there have been long-standing suggestions of a role
for biotin in the regulation of gene expression. For example, biotin has been reported to regulate the synthesis of hepatic glucokinase and to repress phosphoenolpyruvate carboxykinase mRNA in rat liver (11,12). An increase in 6-phosphofructokinase mRNA following biotin administration has been demonstrated in biotin-deficient rats (13,14). Biotin has also been shown to increase the level of the asialoglycoprotein receptor in hepatoma cells using a mechanism that appears to function through cGMP and guanylate cyclase (15–17). More recently, Leon-Del-Rio and colleagues (18) reported that mRNA levels of HCS, ACC1 and the α subunit of PCC were all reduced in biotin-starved human hepatoma cells but could be restored to initial levels by resupplementation with biotin. Significantly, fibroblasts from a patient with HCS deficiency required 100× more biotin than control cells to restore mRNA levels, suggesting a direct role of HCS in the transcriptional response to biotin. These effects were also shown to be mediated through cGMP and guanylate cyclase dependent pathways.

The regulatory role of HCS and biotin in eukaryotic cells are the bases for examining their subcellular distribution in cells.

Biotin has also been reported in the nuclei of cells. Early studies revealed that a large proportion of radioactive biotin, injected into chicks and rats, localized to the nuclear fraction (22), and other studies have reported biotin in nuclei of tumor material and normal tissues (23,24). Recently, Zempleni and colleagues (25) showed that all five histone classes extracted from human peripheral blood mononuclear cells contain biotin. Studies in vitro have suggested that biotinidase may be involved in hydrolysis of biotin from histones and in its synthesis, as well (26–28). However, these studies have not been extended to cells in situ and evidence for nuclear localization of biotinidase is lacking.

In keeping with a nuclear role for HCS, we report here that the majority of the enzyme localizes to the nucleus rather than to the cytoplasm where the carboxylases are found. Our results indicate HCS is associated with chromatin and the nuclear lamina and is distributed in unique ring-like structures during mitosis. We show further that HCS catalyzes the addition of biotin to histones in vitro and that cells from patients with MCD are severely deficient in histone biotinylation. We propose that the role of nuclear HCS is in histone modification which may be linked to the participation of biotin in the regulation of gene expression.

RESULTS

HCS is localized primarily to the nucleus

The subcellular distribution of HCS was determined by immunofluorescent detection using three different antibodies generated against HCS components: anti-HCS, made against the full-length recombinant human protein (residues 58–726); anti-nHCS, made against a N-terminal peptide (residues 58–77); and anti-cHCS, made against a C-terminal peptide (residues 717–726). The presence of HCS was assessed by immunofluorescence in several cell-lines, including HeLa, Hep2, HepG2, CHO-K1, COS and C2C12 myoblasts. All three antibodies detected a punctate distribution of HCS primarily in the nucleus of interphase cells, in addition to weaker staining in the cytoplasm (Fig. 1). Recombinant full-length HCS (FL-HCS), containing the Flag epitope for detection when introduced into HeLa cells by transfection, adopted a similar distribution as endogenous HCS, confirming the identity of the endogenous antigen (Fig. 1). In addition, a cDNA fragment, expressing only the C-terminal half of the protein (C-term-HCS; residues 378–726), also detected with Flag antibody, produced a similar pattern as obtained for the endogenous and full length recombinant proteins. These results indicated that the capacity to target to the nucleus resides in the C-terminal half of the protein, although a recognizable nuclear localization signal was not detected.

Immunoblot analysis with all three antibodies confirmed the results of the immunofluorescence study. When nuclear and cytoplasmic extracts of HeLa and Hep2 cells were examined by Western blot, the three antibodies recognized HCS as two protein species of 66 and 68 kDa (Fig. 2A, showing results for HeLa cell extracts using anti-HCS as antibody). A similar doublet could be detected in the cytoplasmic extract if it was first immunoprecipitated to concentrate the HCS (Fig. 2B). Pre-incubation of all three antibodies with bacterially expressed HCS resulted in the loss of signal demonstrating the specificity of each of the HCS antibodies (Fig. 2C; shown for anti-HCS).

HCS complexes contain biotin

In order to assess whether nuclear HCS is involved with biotin, it was first determined whether it is associated with biotin-containing proteins. To do this, western blots of anti-biotin and anti-HCS immunoprecipitates (IP) of a HeLa cell nuclear fraction were probed with avidin-HRP, to detect biotin or anti-HCS (Fig. 3). The anti-biotin IP contained many biotin-containing proteins, among them protein bands with mobilities consistent with histones (see below), as well as an array of larger proteins (Fig. 3A, left lane). When the same anti-biotin IP was probed with anti-HCS, we detected a single band of 68 kDa (Fig. 3B, left lane). This band aligns with the upper band of the 68 and 66 kDa doublet corresponding to HCS detected in nuclear anti-HCS IPs (Fig. 3B, right lane). This indicates that HCS either contains biotin or was co-immunoprecipitated with a biotin-containing protein. These alternatives were distinguished by probing the anti-HCS IP with avidin-HRP. This procedure also detected the 68 kDa band, indicating that HCS itself contains biotin (Fig. 3A, right lane).

To assess the significance of biotinylated versus unbiotinylated HCS, it was determined if biotinylation of HCS affects its association with other components. Permeabilized HeLa cells, untreated or treated with nocadazole to block the cells at mitosis, were extracted with NP-40 to solubilize non-associated proteins (29), and the recovered supernatants and pellets were examined by western blot for the presence of HCS. Extracts
from cells treated with NP-40, with or without the mitotic block, contained exclusively the lower, non-biotinylated form of HCS (Fig. 3C), while the pellets showed both forms of the enzyme. This observation suggests that the 68 kDa biotin-bound HCS is associated with other species or structures that prevent its extraction with NP-40, while the 66 kDa unbiotinylated HCS remains unassociated and extractable with NP-40. The enrichment of mitotic cells, to over 90% of cells in M phase, produced no difference in the behavior of the two HCS species.

HCS is associated with the nuclear matrix

The subnuclear distribution of HCS in HeLa cells was investigated by performing sequential extraction of soluble, chromatin and nuclear matrix-associated proteins. Immunoblot analysis with HCS antibodies showed that HCS fractionates primarily with chromatin and the core nuclear matrix, although some enzyme could be found in all fractions (Fig. 4). The addition of DNase I did not significantly alter the distribution of HCS. The efficacy of the fractionation procedure was confirmed by the distribution of known nuclear proteins: p38MapK, a stress-activated protein kinase found in the cytoplasm and nucleus; Sp1, a transcription factor associated with chromatin; and lamin B, a nuclear lamina-associated protein (Fig. 4).

Using a similar approach to detect HCS in cells in situ, HeLa cells were grown on coverslips and sequentially extracted with cytoskeletal (CSK) buffer to remove soluble proteins, ammonium sulfate and DNase I to release chromatin proteins and 2 M NaCl, leaving only the nuclear matrix. Each step was monitored by immunofluorescence for detection of HCS as well as lamin B and the nuclear pore complex, known components of the nuclear envelope, for comparison. We found that treatment with CSK buffer removed cytoplasmic HCS but left nuclear HCS in an evenly distributed, punctate pattern (Fig. 5). Following treatment with ammonium sulfate and DNase I, the distribution of nuclear HCS became less even and more concentrated at the nuclear periphery. HCS was still detected after high salt treatment, indicating that a significant proportion of HCS protein is associated with the core nuclear matrix (Fig. 5). Interestingly, the distribution of HCS in the nuclear lamina was discontinuous by comparison with lamin B and the nuclear pore complex. These results are compatible with those from the subnuclear fractionation in that, while HCS is extractable, a significant portion of enzyme remains tightly associated with the nuclear lamina.

HCS localizes to distinct structures in M phase

The localization of HCS to the nuclear lamina led us to characterize its distribution upon nuclear envelope disassembly at the onset of mitosis. In immunofluorescence studies of mitotic cells, HCS signal was excluded from condensed chromosomes (Fig. 6A). It dispersed as bright, large, particulate-like foci from early prophase to late telophase. A more detailed examination of these foci revealed that they have a distinct, ring-like structure irrespective of cell-type or fixation (Fig. 6B). These ring-like structures were found to be quite large, estimated in the order of 3–5 μm in diameter. Up to 98% of HCS was found to co-localize with lamin B, a component of the nuclear lamina (Fig. 7) using Imaris 3.3.1 software (Bitplane) which calculates a statistical distribution of observed signals in three dimensions.

HCS biotinylates histones in vitro and is defective in MCD cells in vivo

To gain insight into the functional state of HCS within the nucleus, nuclear fractions were assayed for biotinylation activity using p67, the biotin attachment domain of propionyl-CoA carboxylase (30), as substrate (Fig. 8). Subnuclear components, produced by stepwise nuclear matrix extraction, were incubated with [14C]-biotin, ATP and p67. As a positive control, bacterially expressed human HCS protein was used as
the source of enzyme. The incorporation of radioactivity into p67 was demonstrated in the nuclear matrix and insoluble fractions, with soluble nuclear components and chromatin showing undetectable activity (Fig. 8). These results are consistent with the distribution of HCS detected with anti-HCS, except in the chromatin fraction, which was inactive but showed presence of HCS by immunodetection. These results show that HCS retains biotinylating activity while associated with the core nuclear matrix, but not as part of the chromatin fraction. Treatment with DNase I did not affect the level or distribution of HCS activity that was observed (Fig. 8).

It was recently shown that histones are biotinylated in human cells in vivo (25). Therefore the capacity of HCS to add biotin to histones was examined. This was first assessed in vitro by incubating purified calf thymus histones and bacterially expressed human HCS in the presence of [14C]-biotin and ATP (Fig. 9A). All histone classes were biotinylated, as was the positive control containing p67 as the substrate. In the absence of HCS, no signal was detected. To assess whether HCS is responsible for histone biotinylation in vivo, the level of biotinylated histones in fibroblasts from MCD patients with deficient HCS activity was examined. Using avidin-HRP or anti-biotin antibodies to detect biotin, immunoreactivity was observed in all histone classes of control cells, confirming that histones are biotinylated in vivo (Fig. 9B). In contrast, histones from patient cells showed a dramatically reduced level of histone biotinylation affecting all five classes. Coomassie staining of isolated histones from control and mutant fibroblasts showed the presence of similar levels of the five major classes of histones (H1, H2A, H2B, H3 and H4; Fig. 9C). These results demonstrate the specific involvement of HCS in histone biotinylation in vivo.

DISCUSSION

We have investigated the distribution of HCS by immunofluorescence in cells in situ and by western blot of fractionated cell extracts during interphase and mitosis in a variety of cultured human cell types, including HeLa, Hep2 and primary skin fibroblasts. The nuclear localization of HCS and its organizational framework in the nuclear lamina and during mitosis point to a nuclear role for this enzyme and its substrate biotin. The ability of HCS to catalyze the biotinylation of histones in vitro and the reduction in histone–biotin content in the cells of patients with HCS deficiency underscore a biological role for HCS and biotin in the nucleus.

Given our traditional understanding of biotin as an essential cofactor of carboxylases and the specific role of HCS in the transfer of biotin to apocarboxylases, it was unexpected to discover that the bulk of immunodetectable enzyme is nuclear. There are several clues as to its function. First, by progressive extraction of nuclear components, HCS was found to be associated with the nuclear envelope, making it one of a growing list of inner nuclear membrane-associated proteins (31). It retained this association even in 2 M salt, typically indicative of a tight association with the nuclear lamina. It behaves similarly to lamin B and to nuclear pore complex in appearing restricted to the nuclear envelope without extending into the ER. However, unlike the uniform distribution of most proteins of the nuclear envelope, HCS is discontinuous, suggestive of a localized deposition of the protein on the surface of the nuclear membrane. While direct membrane association has not been ruled out, it is unlikely given the
absence of a potential membrane-spanning domain, in particular in the C-terminal half of the protein, which was successfully targeted to give a similar distribution as the endogenous protein. Second, the association of HCS in ring-like particles, which colocalize with lamin B, suggests participation by HCS in the dynamics of nuclear envelope assembly-disassembly. Third, attempted disruption of the association of HCS with chromatin using DNase I had minimal effect. While a significant fraction of HCS co-purified with the chromatin fraction, it appears that HCS is at most only loosely attached to chromatin. Fourth, and most significantly, HCS attached to the core component of the nuclear lamina retains biotinylating activity and is capable of biotinylating all five classes of histones in vitro. This was confirmed in fibroblast cultures from patients with deficiency of HCS activity, which showed dramatically reduced biotin content in all five histone classes. These data suggest that the primary role of nuclear HCS is to attach biotin to histones.

Several findings concerning HCS are not readily explained by this study. Biotin is known to attach to a highly restricted biotin binding domain in carboxylases. For most carboxylases, biotin attachment is to the ε amino group of the Lys residue in the sequence AMKM, a core sequence that is conserved in most species at the center of a larger domain of 60–70 amino acids in length. This is the case for the p67 biotin-binding peptide of the α subunit of human PCC used in this study (30). However, there are exceptions, including VMKM (ACC in many species) and AMKL (biotin carboxyl carrier protein in Arabidopsis) as surveyed in genome databases. In addition, biotinylated proteins in plant seeds have been shown to contain novel biotin attachment sequences which have the consensus, (V/M)GKF, with attachment at ‘K’ (32). While histones do not contain a classical biotin-attachment sequence, they have an abundance of Lys residues, any of which could be involved in biotin binding. Such potential is implied by the results of studies using combinatorial libraries to define biotin-attachment sequences. These experiments have demonstrated that both the minimum length and core attachment sequence can vary considerably (33,34), illustrating the potential for variant biotin binding sequences and opening the possibility that histones have a unique biotin attachment site.

Another unexpected finding is the presence of seemingly covalently bound biotin on HCS. This observation has not been made before either for HCS or for BirA. Neither protein has a traditional biotin attachment sequence. Significantly, the electrophoretic mobility of biotin bound and unbound HCS in SDS–polyacrylamide gels shows an apparent molecular mass difference of ~2000 (68 versus 66kDa, in favor of the bound form), suggesting that biotin, a small molecule, has a significant conformational impact by comparison with its unbound counterpart. Clearly, it will be important to determine if the two forms of HCS have the same primary structure or other post-translational modification. A possible explanation for a
self-biotinylated HCS is that the covalently bound biotin may be an intermediate in biotin transfer, a mechanism that would be distinct from biotin transfer to apocarboxylases. There may be precedent for a biotinylated-HCS intermediate by analogy with histone acetylation. ESA1 and Tip60, members of the MYST subfamily of histone acetyltransferases, produce self-acetylation as an intermediate in the transfer of the acetyl-group to the histone lysine (35,36). In ESA1, the acetyl group is attached to a Cys residue which occurs prior to interaction with histone.

The early evidence for nuclear localization of biotin (22–24) and the recent documentation of histone biotin in cells (25) indicate that the function of biotin goes well beyond its role as the prosthetic group of biotin-containing carboxylases. The first suggestion for the attachment of biotin to histones came from the studies of Wolf and colleagues who determined that biotinidase, which releases biotin from biocytin, can transfer biotin to histones in vitro (26). This activity could be demonstrated with purified enzyme and was reduced in the sera of patients with biotinidase deficiency. Similarly, the hydrolysis of histone–biotin to release free biotin is also catalyzed by biotinidase and is reduced in sera or lymphocyte extracts from patients (28). The confirmation of biotin attachment to histones in vitro gave potential significance to these findings, but neither the biotinidase-catalyzed addition nor removal of biotin from histones has been demonstrated in vivo. Furthermore, the requirement for biocytin, a product of proteolysis, as the source of biotin for biotin addition to histones makes it unlikely that this activity occurs in the nuclei of cells. These concerns challenge the biological relevance of biotinidase in histone biotinylation.

Our results suggest that the nuclear biotin-transfer enzyme is HCS. Its substrate is free biotin which is added in the presence of ATP. In the biotinylation of carboxylases, biotin addition occurs via the biotin-5′-AMP intermediate, and we surmise that this is the case for histone biotinylation as well. However, the expected requirement for a substrate sequence comparable to the well-conserved AMKM attachment site, a sequence that is not observed, even remotely, in any of the histones, leaves the mechanism an enigma. This opens the possibility of a novel mechanism that might include a self-biotinylated HCS, as mentioned above. Interestingly, the high abundance of Lys residues in histones has the potential to make available numerous, if variant, sites. At this time, it is not possible to speculate whether core or tail regions of the histones will be biotinylated, although determining this may provide clues as to its function. As well, it will be interesting to determine if HCS and biotinidase act in concert to attach and remove biotin from histones by analogy with their action on apocarboxylases.

The biotinylation of histones adds to the many types of histone modifications that have been documented, including acetylation, phosphorylation, methylation, nitration and ubiquitination (37). Histone modification has been thought to affect gene expression by causing structural alterations in chromatin, thereby affecting nucleosomal access of transcription machinery and linker proteins (38). Alternatively, it has been proposed that multiple, regional histone modifications may result in the generation of a code that is recognized by transcriptional machinery or linker proteins thereby affecting gene expression (37,39). The biological importance of histone biotinylation remains unknown, although biotin deficiency in rats has been reported to be associated with reduced liver RNA, DNA and histone content and reduced histone–DNA interaction (40). It was also shown to affect the level and turnover of histone phosphorylation, acetylation and methylation (41). All of these phenomena could be reversed within days of readministration of biotin to normal levels. Similarly, the impact of biotin on the expression of genes for glucokinase, 6-phosphofructokinase, ACC, PCC and HCS itself all point to a nuclear impact of biotin and a potential role for HCS in mediating its action. Furthermore, the demonstration by Solorzano-Vargas et al. (18) that the transcriptional response to biotin involves a complex signaling cascade that includes HCS and guanylate cyclase activation further underscores the regulatory potential of biotin.

The clinical and biochemical symptoms of MCD patients have been thought to parallel the symptoms associated with the individual carboxylase deficiencies due to the low affinity of mutant HCS for biotin (1). However, symptoms in many patients such as erythematous skin rash and hair loss cannot be explained in this manner. Zempleni and Mock (42) have suggested that marginal biotin deficiency may be teratogenic.

In this light, biotin deficiency may affect the expression of a wider range of genes including developmentally important transcripts in rapidly dividing tissues.
It will be important to determine if histone biotinylation and biotin-mediated transcriptional regulation are linked. Perhaps biotinylation of histones is a general mechanism to control overall gene expression while the guanylate cyclase pathway specifically affects genes involved in biotin utilization and metabolism. Whether by guanylate cyclase or histone biotinylation, HCS seems to be a common factor in both mechanisms and discerning the relationship between these two pathways may sort the novel functions of biotin and HCS. Taken together, our results suggest that HCS, once considered to be a cytoplasmic protein required only to transfer biotin to apocarboxylases, now must be re-evaluated as a protein with an additional role in gene expression or regulation.

**MATERIALS AND METHODS**

**Antibodies**

To obtain HCS antibodies, rabbits were immunized with: (i) purified full length HCS fusion protein containing residues 58–726 (43), resulting in anti-HCS; (ii) N-terminal peptide containing HCS amino acids 58–78 (Sheldon Biotechnology Centre, Montreal, Canada), resulting in anti-nHCS; and (iii) C-terminal peptide containing HCS amino acids 706–726 (Sheldon Biotechnology Centre, Montreal, Canada), resulting in antiCHCS. Other primary antibodies used included monoclonals: anti-p38MapK (BD Biosciences), anti-Sp1 (BD Biosciences), anti-LaminB (Oncogene), anti-NPC (nuclear pore complex mAbb414; Babco), anti-Flag (Sigma), anti-biotin and anti-biotin agarose (Sigma).

**Plasmid construction**

Full-length HCS cDNA (residues 58–726) and C-terminal HCS cDNA (residues 378–726) (43) were each subcloned into the EcoRI site of pCMV-Tag4 (Stratagene) to generate FL-HCS and C-term-HCS in-frame with a C-terminal Flag-tag, the latter for detection in cells in situ using anti-Flag antibody.

**Cell-culture and synchronization**

HeLa (human cervical adenocarcinoma), Hep2 (human laryngeal carcinoma), HepG2 (human hepatoma), CHO-K1 (hamster ovarian), COS (monkey kidney) and C2C12 (mouse myoblasts) cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum and antibiotics. Normal human fibroblasts, MCH39 and MCH64, and MCD patient fibroblasts, MCD-VE and MCD-MK (Montreal Children’s Hospital Cell Repository, Montreal, Canada) were cultured as described previously (5). To synchronize cells at M phase, cells were incubated overnight with 0.1 µg/ml of nocodazole (Sigma). The cells were centrifuged, plated on coverslips and incubated in culture medium for 6 h prior to immunofluorescence or other analysis.

**Immunofluorescence**

Cells collected from a confluent 10 cm² plate were scraped into 1 ml of PBS and collected by microfugation at full speed for 5 min. Nuclear and cytoplasmic extracts were prepared using NE-PER nuclear and cytoplasmic extraction kit as recommended (Pierce). Protein concentrations were determined using Bradford reagent (Bio-Rad). Cross-contamination between fractions was monitored by western analysis of cytoplasmic and nuclear extracts using mAbs anti-p38MapK and anti-Sp1, respectively.

**Immunoprecipitation and immunoblot analysis**

Cell extracts were precleared by incubation overnight at 4°C with normal rabbit serum and protein A-Sepharose (Pharmacia). Precleared extracts were recovered by microfugation and immunoprecipitated with anti-HCS or preimmune sera for 4 h at 4°C prior to overnight incubation with protein A-Sepharose. Alternatively, cell extracts were incubated with anti-biotin agarose (20 µl for 100 µl cytoplasmic or nuclear extract) overnight at 4°C. IPs were washed four times with cold...
PBS, extracted by heating at 95°C for 5 min in SDS sample buffer and resolved by SDS–PAGE.

Cell lysates (30 μg) or IPs were resolved by discontinuous SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane by capillary action. The membranes were blocked with 5% dry milk overnight and then incubated with antibody (anti-HCS, anti-nHCS or anti-cHCS at 1:100; anti-p38MapK at 1:1000; anti-Sp1 at 2 μg/ml; anti-Lamin B at 5 μg/ml; anti-Biotin at 1:500 or anti-Flag at 1:1000) for 1 h. For preadsorption of HCS antibodies, 5 μg of antibody was incubated with 5 μg of bacterially expressed HCS protein overnight at 4°C with tumbling. Immune complexes were detected using HRP-conjugated anti-rabbit or anti-mouse IgG (BioRad) and HRP coloured reagent (BioRad) as recommended. Biotin-containing proteins were detected using avidin-HRP (BioRad) at 1:2000. Polyacrylamide gels or protein blots were analyzed using the GelDoc gel documentation system and GelSnap 4.0 software (SynGene, Cambridge, UK).

**In vitro nuclear matrix isolation**

Fractionation of nuclear matrix proteins from HeLa cells was adapted from Qiao et al. (44). Briefly, cultured cells collected from a confluent 10 cm² plate were resuspended in 5 ml of low-salt buffer (10 mM Hepes, pH 7.4, 10 mM KCl, 50 μg/ml digitonin and protease inhibitors) for 15 min at 4°C. Permeabilized nuclei were recovered by centrifugation at 1000 rpm for 5 min at 4°C. The soluble protein supernatant was recovered and nuclei were washed twice using low salt buffer and resuspended in 200 μl of low salt buffer with or without 30 units of DNase I for 1 h at 4°C. Chromatin proteins were extracted by adding 200 μl of extraction buffer (1% Triton-X, 50 mM Hepes, pH 7.4, 150 mM NaCl, 30 mM Na₃P₂O₇·10H₂O, 10 mM NaF, 1 mM EDTA and protease inhibitors) for 10 min at 4°C. Supernatant was collected by microfuging for 10 min. The pellet was solubilized in an equal volume of urea buffer (8 M urea, 100 mM NaH₂PO₄ and 10 mM Tris, pH 8.0) for 30 min at 4°C.

**In situ nuclear matrix extractions**

HeLa cells, seeded onto four glass coverslips, were sequentially treated based on method of Berube et al. (45) with some modifications. One coverslip was left as an untreated control. The remaining coverslips were washed twice with PBS and treated with CSK buffer (0.5% Triton-X, 10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EDTA, 1 mM PMSF, 1 mM DTT and protease inhibitors) for 10 min at 4°C. A coverslip was set aside representing the elimination of soluble proteins. The remaining coverslips were treated with 1 M ammonium sulfate supplemented with 100 μg/ml of DNase I for 5 min at room temperature. A coverslip was set aside representing the loss of chromatin proteins. The remaining coverslip contained only core nuclear matrix following incubation in a high-salt wash (2 M NaCl in CSK buffer) for 5 min at 4°C. All coverslips were analyzed by immunofluorescence.

**HCS activity assays**

Cell extracts were tested for their ability to biotinylate peptide substrate fragment p67 as described (30). Briefly, 50 μg of cell extract or 25 μg of bacterially expressed HCS were mixed with 5× biotinylation buffer (250 mM reduced glutathione, 125 mM MgCl₂ and 1.5 M Tris, pH 7.5), 1 μl of 100 mM ATP, 5 μM p67 or 5 μg of calf-thymus histones (Boehringer-Mannheim) and 0.05 μCi of [14C]-biotin (Amersham) to a final volume of 20 μl. Following overnight incubation at 37°C, the reaction was stopped by the addition of an equal volume of SDS sample buffer. Samples were heated to 95°C for 5 min and electrophoresed on a 16.5% Tris–tricine gel. Proteins were transferred onto a nitrocellulose membrane by western blotting, exposed to a phosphorimaging plate and processed to visualize radioactive bands using the accompanying software (Molecular Dynamics).

**Histone extraction**

Histones were extracted as described by Stanley et al. (25) with some modifications. Cells scraped from a confluent 10 cm² dish were resuspended in 200 μl of HNB (0.5 M sucrose, 15 mM Tris, pH 7.5, 60 mM KCl, 0.25 mM EDTA, pH 8.0, 0.5 M spermidine, 1 mM DTT and protease inhibitors) followed by the dropwise addition of 100 μl of HNB supplemented with 1% NP-40. Cells were incubated for 5 min while monitoring cytoplasmic dissociation microscopically. Nuclei were pelleted by microfugation at 6000 rpm for 3 min. Nuclei were resuspended in 50 μl of 1 M HCl containing 2% 2-mercaptoethanol and 2 mM PMSF. The suspension was incubated overnight at 4°C to extract histones followed by centrifugation at 10 500 rpm for 4 min. The supernatant was saved and the pellet resuspended in 50 μl of the 1 M HCl solution for another 6 h at 4°C. Centrifugation was repeated and both supernatants were combined. The pH was adjusted to 7.0 using 3 M KOH.

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**REFERENCES**


24. Yokoyama, S., Kashima, K., Inoue, S., Daa, T., Nakayama, I. and Moriuchi,

5. Dupuis, L., Leon-Del-Rio, A., Leclerc, D., Campeau, E., Sweetman, L.,