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Nutritional regulation of nucleosomal structure at the chicken malic enzyme promoter in liver

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ABSTRACT

Transcription of the chicken malic enzyme gene in the liver is stimulated by feeding and inhibited by starvation. Concomitant with the increase in transcription caused by refeeding, chromatin structure around the transcription start site of the malic enzyme gene is modified in the liver. Digestion of chromatin in isolated nuclei with DNase I revealed four feeding-induced DNase I hypersensitive sites (−220, −170, −130 and −70 bp) near the malic enzyme promoter. Similarly, digestion of chromatin with restriction endonucleases detected enhanced cleavage within this region when birds were refed. Micrococcal nuclease detected the presence of nucleosomes over this region in the starved state, but not in the fed state. After food was withdrawn from fed birds, nucleosomes were reformed in this region within 6 h. The speed and magnitude of the changes in nucleosomal structure in this region suggest that they did not require DNA replication.

INTRODUCTION

In mammals and birds, the state of alimentation regulates the concentrations of several hepatic enzymes involved in energy metabolism (reviewed in ref. 1). This allows the animals to adjust the rates of metabolic fluxes such that energy in excess of current caloric requirements can be stored when food is abundant, and glucose homeostasis can be maintained even when food is temporarily unavailable. One of these enzymes, cytosolic malic enzyme (L-malate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.40) furnishes NADPH for fatty acid biosynthesis. The concentration of malic enzyme is 70-fold higher in liver of fed chicks than in that of starved ones, primarily as a result of transcriptional regulation (2).

To gain some insight into the mechanism by which transcription of the malic enzyme gene is regulated by nutritional status, we have analyzed the effect of nutritional status on the chromatin structure around the 5′-flanking region of this gene (2). Refeeding starved chicks greatly enhances DNase I hypersensitivity of the 400-bp region immediately upstream of the transcription start site of the malic enzyme gene in hepatic nuclei. The increase in DNase I hypersensitivity occurs within 3 h of refeeding, coincident with the onset of stimulated transcription. This change in chromatin structure does not occur in non-hepatic tissues, where transcription of the malic enzyme gene is low and not stimulated by refeeding. Structural and functional characterization of this region indicates that it contains the promoter for the malic enzyme gene (S.A.Klautky, D.A.Fantozzi, and A.G.Goodridge, unpublished results).

One significant aspect of inducible DNase I hypersensitive sites is that they often encompass cis-acting DNA elements which are the targets of inducing stimuli. Glucocorticoid-induced DNase I hypersensitive sites, for example, contain glucocorticoid response elements (GREs) (3–5). Although several genes are regulated by nutritional status, the cis-acting DNA elements that mediate this response have not been identified. Therefore, we wished to localize DNase I hypersensitive sites induced by feeding at the malic enzyme promoter. Furthermore, we wanted to investigate the structural basis for the differential DNase I sensitivity of the malic enzyme promoter under different nutritional conditions. The dynamics of chromatin structure may be involved in regulation of transcription (6–9).

In this study, we employed DNase I, restriction endonucleases and micrococcal nuclease to probe chromatin structure at the malic enzyme promoter under different nutritional conditions. Feeding induced DNase I hypersensitivity at four sites (−220, −170, −130 and −70 bp) within the proximal region of the malic enzyme promoter. Micrococcal nuclease detected the presence of nucleosomes in the region spanning +72 to −320 bp in the starved state, but not in the fed state. Nucleosomal structure in this region was rapidly disrupted by feeding, and rapidly reestablished during starvation, suggesting that these chromatin transitions did not require DNA replication.

MATERIALS AND METHODS

Animal care

Except as noted, white Leghorn chicks (2–3 weeks old) were fed commercial chicken mash ad libitum.

Isolation of nuclei and digestion with DNase I

Nuclei were isolated from livers of chicks that were starved for 48 h or starved for 48 h and then refed for 6 h. DNase I digestion was carried out as previously described (2).

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Isolation of nuclei and digestion of chromatin with restriction endonucleases and micrococcal nuclease

Nuclei were isolated (10) and portions (100 μl) of nuclei containing 5 OD_{260} units (as estimated by lysing nuclei in 1% SDS) were used for nuclease digestion. To suppress endogenous endonuclease activities, incubations with restriction enzymes and micrococcal nuclease were carried out on ice.

Incubation with restriction endonucleases was in 10 mM Tris, pH 7.4, 15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM DTT, 5% glycerol for one hour. Digestion was stopped by adding EDTA to 10 mM. Digestion with micrococcal nuclease was at the indicated concentrations in 10 mM Tris, pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 0.25 M sucrose and 1 mM $CaCl_2$ for 20 min and then stopped by 10 mM EDTA and 1 mM EGTA (11).

Nuclease-treated nuclei were treated with Proteinase K, and the DNA extracted with phenol/chloroform. The extracted DNA was precipitated with ethanol, and dissolved in 10 mM Tris, pH 8.0, 1 mM EDTA.

Analysis of DNA

DNA from nuclei treated with restriction enzymes or DNase I was further digested with PmlI or XhoI as indicated. PmlI digested DNA (10 to 20 μg) was subjected to electrophoresis on 3% agarose gels (3 parts of NuSieve : 1 part of Seakem agarose, FMC). DNA digested with XhoI was analyzed on 1.4% agarose gels. DNA (10 μg) from nuclei treated with micrococcal nuclease was fractionated on 1.8% agarose gels. DNA was transferred from agarose gels onto Nytran membranes (Schleicher & Schuell) using the VacuGene apparatus (LKB Pharmacia). The membrane was baked for 30 min at 80°C and then irradiated with UV to immobilize small fragments according to the manufacturer's instructions (Schleicher & Schuell).

The DNA probes used in this study were derived from genomic clones for malic enzyme obtained in this laboratory (D.Fantozzi and A.G.G., unpublished results). A PstI fragment containing the malic enzyme promoter region (−418 to +222 bp) was subcloned into the polylinker region of the plasmid vector pIBI31 (International Biotechnologies, Inc.). The RsaI/PstI fragment (+4 to +222) and the BglI fragment (−209 to +62) were excised from this plasmid DNA by the indicated restriction endonucleases. The intronic probe (ME-C3) for the malic enzyme gene was previously described (2). ^{32}P-Labeled probes were prepared by the method of Feinberg and Vogelstein (12). Hybridization was carried out in the presence of 10% dextran sulfate (2). The final wash was in 0.1×SSPE (18 mM NaCl, 1 mM $NaPO_4$, pH 7.2, 0.1 mM EDTA) plus 0.5% SDS at 68°C for 30 min. The resulting membrane was exposed to X-ray film (X-OMAT AR, Kodak) for 2 to 5 days at −70°C.

DNase I hypersensitive sites *in vitro*

Nuclear extracts from chicken livers were prepared using 0.6 M KCl in the extraction buffer (13). Protein concentration was determined by the Bradford method (14) using bovine serum albumin (Sigma) as standard. Plasmid DNA containing malic enzyme sequences from −418 to +222 was digested with KpnI and HindIII. The KpnI/HindIII fragment was isolated from agarose gels by eletroelution, and radiolabeled at the HindIII site by the Klenow fill-in reaction (15). Nuclear extracts were adjusted to a volume of 100 μl with 10 mM Tris, pH 7.4, 15mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM dithiothreitol, 5% glycerol. Labeled KpnI/HindIII fragment (0.5 ng) and poly(dIdC)-poly(dIdC) (3 μg) were added, and the mixture was incubated on ice for 40 min. DNase I was then added to the indicated concentrations and incubation continued for 10 min on ice. Digestion was stopped by 10 mM EDTA and phenol-chloroform extraction. Products were analyzed on 6% non-denaturing polyacrylamide gels. Gels were dried and exposed to X-ray film (X-OMAT AR, Kodak) for 2 to 3 days.

RESULTS

DNase I hypersensitive sites in the promoter region

In chromatin of chicken liver, the malic enzyme promoter becomes much more sensitive to DNase I when starved birds are refed (2). Here, we wished to map the DNase I hypersensitive sites at the malic enzyme promoter at higher resolution. Nuclei from livers of chicks that were starved for 48 h or starved for 48 h then refed for 6 h were treated with DNase I, and DNA was purified. The sites of DNase I cleavage were mapped by the indirect end-labeling technique (16). DNase I-treated DNA was digested with PmlI, and fractionated on 3% NuSieve agarose gels. To improve size estimations, genomic DNA of high molecular weight was partially digested with DdeI and then further digested with PmlI and size-fractionated on the same gels. The probe for indirect end-labeling was the 219-bp RsaI/PstI fragment (+4 to +222 bp). Five major sites of DNase I cleavage were detected. Using DdeI sites within this region (confirmed by sequencing, data not shown) as references, DNase I cleavage sites were mapped at −320, −220, −170, −130 and −70 bp (±10 bp) (Fig. 1). DNase I cleavages at and upstream from −320 bp were similar in intensity between starved and refed samples. The four downstream sites were greatly induced by refeeding, indicating a highly localized transition in chromatin structure.

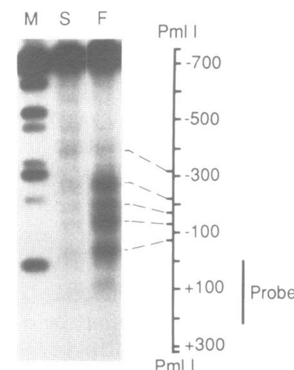

Figure 1. High resolution mapping of DNase I hypersensitive sites at the malic enzyme promoter. Nuclei from livers of chicks starved for 48 h or starved for 48 h then refed for 5 h were treated with DNase I (0.2 U/ml) for 10 min at 37°C. DNA (20 μg) from starved (lane S) or refed (lane F) chicks was digested with Pml I, separated by size on a 3% NuSieve agarose gel, and transferred onto Nytran membrane (Schleicher & Schuell). High molecular weight genomic DNA was partially digested with DdeI and then completely digested with PmlI, and run on the same gel (lane M); DdeI sites within the malic enzyme promoter region were used as references. DNase I and DdeI fragments were detected by indirect end-labeling (16) using an RsaI/PstI probe (+4 to +222). Major DNase I hypersensitive sites were mapped to −320, −220, −170, −130 and −70 bp (±10 bp).

Promoter accessibility to restriction enzymes

Restriction endonucleases have been used to probe chromatin structure (17, 18). Regions of chromatin hypersensitive to DNase I are also hypersensitive to restriction endonucleases. In addition to complementing the DNase I data, restriction endonucleases should help to delineate the boundaries of the chromatin transition.

We used Dde I and Hae III to digest chromatin in isolated nuclei and compared the patterns of digestion between nuclei from starved (24 h) or continuously fed chicks. The restriction cleavages were displayed by indirect end-labeling, employing the same strategy used to map DNase I hypersensitive sites. For DdeI, three sites (−50, −180, −245) were cleaved with less efficiency in the starved samples than in the fed ones, whereas sites at −404 and further upstream were digested to similar extents in both sets of samples (Fig. 2A). The same result was obtained when a probe derived from the other end of the PmlI fragment was used for indirect end-labeling (data not shown). Similarly, HaeIII digestion at −200 was inhibited in the starved samples (data not shown). To determine whether sites further upstream from −400 were hypersensitive to nucleases in hepatic nuclei from fed birds, we used a −780/−450 bp probe for indirect end-labeling. Sites upstream from −400 were not more sensitive to nucleases in the fed state than in the starved state (data not shown). Thus, using both DNase I and restriction enzymes, the increase in nuclease sensitivity in the fed state was localized to the domain from −320 to +72 bp (Fig. 2B).

Micrococcal nuclease digestion

What is the structural basis for the differential nuclease sensitivity in the region between −320 and +72? One possibility is that this differential nuclease sensitivity indicates altered nucleosomal structures over this region under different nutritional states. The size of this domain is about 400 bp, consistent with the possibility that it is associated with two nucleosomes in the starved state. Micrococcal nuclease attacks preferentially the linker regions between nucleosomes such that chromatin consisting of a polynucleosomal array will be digested into fragments containing one, two and more nucleosomes. DNA purified from these chromatin fragments will have lengths corresponding to multiples of the nucleosomal repeat (about 180 bp). Nucleosome-free DNA should have a more diffuse size distribution.

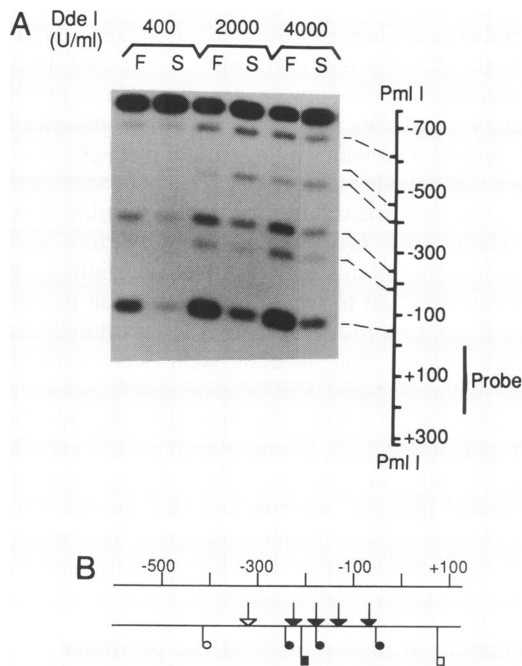

Figure 2. Accessibility of the proximal region of the malic enzyme promoter to restriction endonucleases. **A.** Nuclei from livers of chicks starved for 24 h or fed continuously were treated with DdeI at the indicated concentrations for 1 h on ice. DNA (10 μg) from starved (lanes S) or fed (lanes F) chicks was digested with PmlI to completion, separated on a 3% NuSieve agarose gel and transferred onto Nytran membrane (Schleicher & Schuell). The pattern of Dde I cleavages was detected as described in the legend to Fig. 1. The DdeI sites are shown in the diagram on the right. **B.** Summary of nuclease digestion data. The scale of the drawing is shown at the top. The numbers refer to positions relative to the transcription start site of the malic enzyme gene. DNase I hypersensitive sites are depicted by arrows. An open arrow indicates a site not affected by nutritional status, whereas the solid arrows indicate sites that were induced by feeding. Circles and squares mark the positions of DdeI and Hae III sites, respectively. Solid symbols indicate cleavages that were repressed in the starved state; the open symbols indicate cleavages that were unaffected by nutritional status.

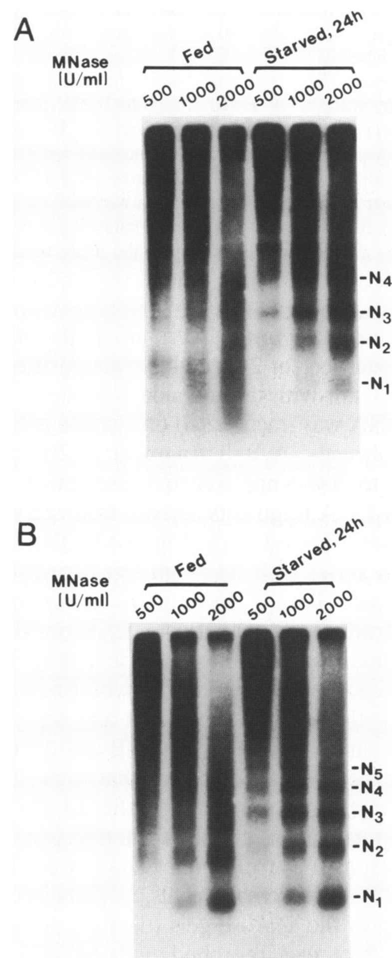

Figure 3. Analysis of the proximal region of the malic enzyme promoter with micrococcal nuclease. Nuclei were isolated from livers of chicks that were starved for 24 h or fed continuously and treated with micrococcal nuclease at the indicated concentrations for 20 min on ice. DNA (10 μg) was separated by size on a 1.8% agarose gel and blotted onto Nytran membrane (Schleicher & Schuell). **A.** Hybridization with the BglI fragment (−209 to +65 bp). Sizes of fragments corresponding to DNA associated with one, two or more nucleosomes are marked N1−N4. **B.** The same membrane was stripped of the first probe and rehybridized with ME-C3 (2), a 1-kb fragment derived from the second intron of the malic enzyme gene.

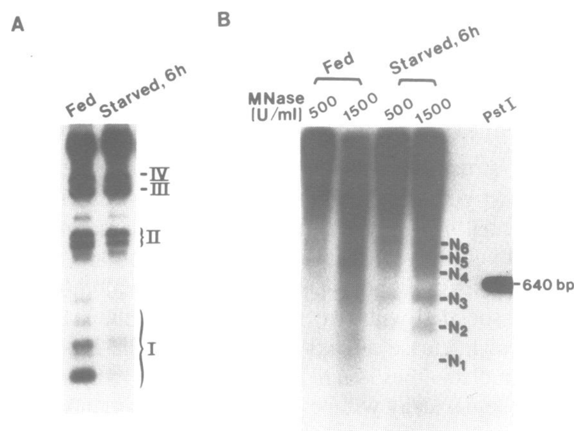

Figure 4. Time course of the transition in chromatin structure at the proximal region of the malic enzyme promoter after withdrawal of food. Nuclei were isolated from livers of chicks starved for 6 h or fed continuously and used for chromatin analyses. **A.** Nuclei were treated with the restriction endonuclease DdeI at 400 units/ml for 1 h on ice. Purified DNA (10 μg) was digested with XhoI and analyzed by the indirect end-labeling technique on a 1.4% agarose gel. The probe was described in the legend to Fig. 1. The upstream XhoI site is at about −10 kb. DdeI hypersensitivity was also observed at regions II−IV in nuclei from both fed and starved birds as noted previously with DNase I (2). **B.** Nuclei were treated with micrococcal nuclease for 20 min on ice at the indicated concentrations. Purified DNA (10 μg) was separated by size on a 1.8% agarose gel and blotted onto Nytran membrane (Schleicher & Schuell). Hybridization was with the probe described in the legend to Fig. 3A. The right-most lane contained genomic DNA (1 μg) digested with PstI to ascertain the specificity of the probe used for analysis of nucleosomes.

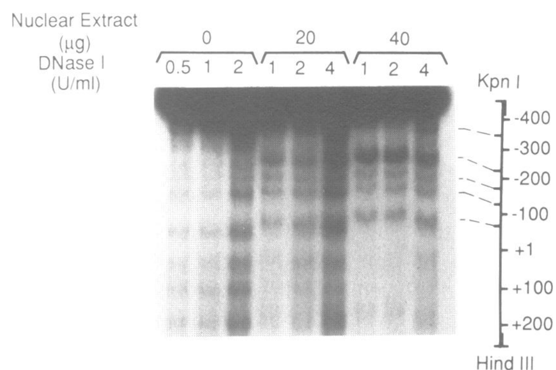

Figure 5. Hepatic nuclear extracts induce DNase I hypersensitive sites in the proximal region of the malic enzyme promoter in vitro. The KpnI/HindIII fragment (−418 to +222 bp) was labeled with ^{32}P at the HindIII site. Both ends had about 30 bp derived from vector DNA; these are indicated by the thicker lines in the drawing on the right. This labeled fragment (0.5 ng) was incubated with different amounts of hepatic nuclear extracts from fed chicks, and then digested with DNase I for 10 min on ice at the indicated concentrations. The purified DNA was analyzed on a 6% non-denaturing polyacrylamide gel, and the dried gel was subjected to autoradiography. Upstream of the transcription start site, there were five major sites of DNase I cleavage (indicated on the right) located at −320, −220, −180, −140 and −80 bp (± 10 bp), respectively.

Nuclei were isolated from livers of birds that were fed continuously or starved for 24 h. DNA was purified from these nuclei after treatment with three concentrations of micrococcal nuclease. The DNA was fractionated on agarose gels, and blotted onto nylon membranes. A BglI fragment (−209 to +62) was used as a probe to detect nucleosomal association. The pattern of distribution of DNA fragments derived from starved birds was different from that from fed birds (Fig. 3A). DNA from starved birds exhibited a series of bands with sizes consistent with the lengths of DNA associated with one, two or more nucleosomes, whereas DNA from fed birds had a much more diffuse length distribution, including some that were less than 100 bp. The specificity of this probe was checked by hybridization to genomic DNA digested with restriction enzymes and separated by size on the same gels and membranes (Fig. 4B and data not shown). Two control experiments indicated that this difference in association of nucleosomes was specific to the −209/+62 fragment. First, total genomic DNA from micrococcal nuclease-treated nuclei originating from either starved or fed birds had the same pattern of nucleosomal repeats, as visualized by ethidium bromide staining of the agarose gels (data not shown). Second, when the same blot was reprobed with ME-C3 (2), a 1-kb fragment derived from the second intron of the malic enzyme gene, the patterns of nucleosomal repeats were similar between fed and starved samples (Fig. 3B).

When the malic enzyme promoter region was analyzed for nucleosome positioning by the indirect end-labeling procedure, the patterns of cutting by micrococcal nuclease were similar between starved and fed samples, which were in turn similar to that of free DNA (data not shown). The similarity between micrococcal nuclease patterns in the indirect end-labeling experiment between free DNA and chromatin from either starved or fed chicks does not necessarily conflict with the differences observed in the direct probe experiment, but could mean that precisely positioned nucleosomes were absent from this region in both dietary conditions. The presence of nucleosomes is not necessarily synonymous with the presence of precisely positioned nucleosomes. On the other hand, however, micrococcal nuclease prefers AT-rich sequences, and the malic enzyme promoter region is about 70% GC. Consequently, cutting sites for micrococcal nuclease in this region of DNA are infrequent and weak, and thus may fail to reveal nucleosomes in this type of experiment. Discrepant results derived from direct hybridization (Fig. 3) and indirect end labeling also were obtained in experiments with the yeast PHO8 gene; nucleosomes at the inactive promoter were detected by direct hybridization, but not by indirect end-labeling(19). The explanations and significance of these observations are unclear in both cases. Based on the results presented thus far, we conclude that the domain from −320 to +72 is associated with nucleosomes in the starved state, and that this nucleosomal structure is disrupted or altered in the fed state.

Rapid formation of nucleosomes at the promoter

DNase I hypersensitivity of the malic enzyme promoter region is maximal within 3 h of refeeding (2). We next asked whether the reverse transition was similarly rapid. We repeated the chromatin analyses with chicks that were starved for different lengths of time. At 6 h after withdrawal of food, the malic enzyme promoter region was insensitive to DdeI (Fig. 4A) and DNase I (data not shown), and was associated with nucleosomes as assayed by micrococcal nuclease (Fig. 4B). In the experiment shown in Fig. 4A, the parent fragment was generated by digestion with XhoI, which cleaves the 5′ flanking DNA of the malic enzyme gene at −10 kb. Dde I hypersensitivity also was observed at regions II−IV in nuclei from both fed and starved birds, as noted previously with DNase I (2). At 4h after food withdrawal,

nucleosomal structure at the proximal region of the malic enzyme promoter was reestablished in some animals but not in others(data not shown). We attribute this variability to the difficulty of synchronizing the initiation of starvation. These animals feed periodically throughout the daylight hours. Therefore, the time between the last food intake and our removal of the food will vary from bird to bird. Nevertheless, during the transition from the fed to starved state, nucleosomal structure at the malic enzyme promoter changed to that characteristic of the starved state within 6 h of food deprivation. Transcription of the malic enzyme gene was decreased to the basal level within 4.5 h after initiation of starvation (2).

DNase I hypersensitivity *in vitro*

The presence of nucleosomes over the proximal region of the malic enzyme promoter in the starved state may explain the low sensitivity of this region to nucleases. However, in the putative nucleosome-free state in liver of fed chicks, the promoter region was not uniformly sensitive to DNase I. Instead, it exhibited discrete sites of DNase I cleavage. The possibility that this pattern of DNase I digestion reflected DNA-protein interactions within this region was tested in vitro. Nuclear extracts were prepared from livers of starved (24 h) or fed chicks. The KpnI/HindIII fragment (−418 to +222) was labeled at the HindIII site, incubated with different amounts of nuclear extract, digested with DNase I at limiting concentrations, and the resulting fragments separated on non-denaturing polyacrylamide gels. In the presence of nuclear proteins from fed chicks, there were a series of five sites over the 400-bp region immediately upstream of the transcription start site, and they were mapped at −320, −220, −180, −140 and −80 bp, respectively (Fig. 5). Within the resolution of these gel systems, the locations of these sites agreed with those of DNase I hypersensitive sites in chromatin in fed chicks. Compared with free DNA, cleavage at −220 bp was enhanced by nuclear proteins. Sequence-specific binding of nuclear proteins often causes deformation of the adjacent DNA structure, facilitating cleavage by DNase I (20). The DNase I hypersensitive site at −220 may represent such a case. Nuclear extracts prepared from starved or fed animals yielded the same result (data not shown), suggesting that proteins capable of interacting with this region were present in both starved and fed states.

DISCUSSION

In this study, we analyzed chromatin structure in the 5′-flanking region of the malic enzyme gene. Our results suggest that in the starved state, the region from −320 to +72 bp was organized into nucleosomes, and that these nucleosomes were disrupted or altered in the fed state. The two states of chromatin structure in this region are readily reversible. When starved chicks are refed, nucleosomal disruption occurs within 3 h (2). Conversely, nucleosomes (as detected by micrococcal nuclease) are formed within 6 h after food is withdrawn from fed birds (Fig. 4). The speed and magnitude of these transitions suggests that DNA replication is not required. Glucocorticoid-induced changes in nucleosomal structure of the rat tyrosine aminotransferase gene also are independent of replication (21). Participation of DNA replication in changes in chromatin structure was discussed in a recent review (22). The mechanism by which replication-independent changes in chromatin structure occur is unknown.

The putative nucleosome-free state of the malic enzyme promoter in the fed state was hypersensitive to DNase I only at discrete sites. In the in vitro DNA binding experiment (Fig. 5), the same set of DNase I cleavages were observed when a cloned fragment was incubated with nuclear extracts. As compared with protein-free DNA, cutting at −220 was enhanced by protein binding. Thus, the pattern of DNase I cleavages in chromatin in the fed state may have two causes: (1) exposure of DNA upon nucleosomal disruption and (2) conformational changes in DNA caused by protein-DNA interactions. The glucocorticoid-induced DNase I hypersensitivity over the MMTV promoter is caused by both DNA exposure due to nucleosomal disruption and protein binding (23). Protein binding-induced DNase I hypersensitivity can be a good marker for protein occupancy in chromatin *in situ* (24). Functional analysis of the DNase I hypersensitive sites upstream of the transcription start site for malic enzyme is in progress.

Reports of changes in chromatin structure caused by nutritional status are rare, but not unique to the malic enzyme gene. Feeding a high carbohydrate diet induces DNase I hypersensitive sites upstream of the S14 gene in rat liver (25). The S14 gene codes for a protein of unknown function, but, because its regulation is coordinated with that of the lipogenic genes, it may be involved in lipogenesis. The paucity of data on changes in chromatin structure as a function of nutritional state make it impossible to know whether the changes that we observe are liver-specific for a majority of genes expressed in liver.

The relationship between the nucleosomal organization of the malic enzyme promoter and transcriptional regulation of this gene is unknown. The increase in DNase I hypersensitivity of the malic enzyme promoter correlated temporally with the increase in transcription when starved chicks were refed (2). Conversely, nucleosome formation at the malic enzyme promoter occurred in a time period similar to that required for complete inhibition of transcription of the malic enzyme gene during the transition from the fed to starved state (Fig. 4). The use of intact animals makes it difficult to determine whether changes in chromatin structure precede or follow changes in transcription rate. Future studies using primary hepatocytes in culture may resolve this question.

ACKNOWLEDGMENTS

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