Binding of HMG 17 to mononucleosomes of the avian β -globin gene cluster in erythroid and non-erythroid cells

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ABSTRACT

The binding of HMG 17 to stripped core mononucleosomes containing DNA from the avian β -globin gene cluster was examined to determine whether binding *in vitro* in this developmentally-regulated gene domain was associated with transcriptional activity or DNaseI-sensitivity in intact nuclei. Mononucleosomes were prepared from primitive and definitive stage embryonic red blood cells of chick embryos, adult reticulocytes, adult reticulocytes in which embryonic ρ -globin transcription was induced, and adult thymus cells. Preferential binding by HMG 17 to mononucleosomes containing the β -globin gene cluster was confined to erythroid-derived mononucleosomes that contain the embryonic ρ -globin gene, the adult β -globin gene, and DNA sequences located between these two genes, but not to those that contain the embryonic ϵ -globin gene. Comparison of these results to the known patterns of transcription and DNaseI-sensitivity within the β -globin gene cluster shows that HMG 17 binding, although tissue-specific, does not correlate directly with either DNaseI-sensitivity or active gene transcription, but is dependent on other factors present in core mononucleosomes from this active gene domain.

INTRODUCTION

The function(s) of the HMG (high mobility group) proteins are not yet established. It has been suggested that the subclass of smaller HMG proteins which includes HMG 14 and HMG 17, may alter the structure of transcriptionally active chromatin. This hypothesis is based on the finding that these proteins can mediate the increased sensitivity to DNaseI-digestion characteristic of transcriptionally active gene domains (1,2). We (3) and others (4,5,6,7) have shown that these proteins bind preferentially to nucleosomes that contain active genes both *in vitro* and *in vivo*. HMG 17-binding to active chromatin has also been reported to be confined to the transcribed regions of active gene domains (8). This finding suggests that HMG 14 and 17 proteins may not be the principle mediators of DNaseI-sensitivity of active gene

domains as this sensitivity often extends well beyond regions of transcriptional activity.

The purpose of this study was to map the extent of HMG 17 binding in the well-characterized avian β -globin gene cluster. Within this cluster are four globin genes; two definitive, adult-stage specific genes, β and β^H , flanked by two embryonic genes, ρ and ϵ (see Fig. 1) (9,10). The embryonic globins are transcribed only in red cells of the primitive, yolk sac-derived stage of erythropoiesis (11,12,13). The rate of transcription of the ρ -globin gene is two to five-fold higher than that of the ϵ -globin gene (11,13). On day 5 of embryogenesis, the only cells found in circulation are those of the primitive stage (11). Erythrocytes of the definitive stage begin to replace the primitive red cells within a few days; this process is complete by day 14 of embryo development. Reticulocytes from day 14 embryos transcribe both β and β^H globin (11,13); in the adult, only β -globin is transcribed (11). Nuclease digestion studies of day 5 and day 14 erythroid cells have shown that the entire β -like globin cluster is DNaseI-sensitive in both cell types (12,14,15). Decreased DNaseI-sensitivity for the embryonic ϵ -gene, relative to β -globin has been reported in day 14 cells (12). However, more recent studies document uniform sensitivity throughout the entire domain (15). Similar studies have not been carried out in adult reticulocytes, although increased DNaseI-sensitivity has been reported for both the ρ -globin and the β -globin genes (16). Lastly, in addition to the developmental switch in globin gene expression, it is possible to activate embryonic ρ -globin transcription (but not ϵ -globin) by treatment of anemic adult chickens with 5-azacytidine and butyrate (16,17).

In this paper, we have examined HMG 17-binding to core mononucleosomes, prepared from the β -globin chromatin domain of day 5 primitive and day 14 definitive chick embryo red blood cells, reticulocytes from anemic adult birds and birds treated with 5-azacytidine and sodium butyrate and adult thymus glands. We have found that the binding of HMG 17 to these mononucleosomes was the same in all erythroid cell populations studied. Erythroid cell derived core mononucleosomes that contain the embryonic ρ -globin and adult β -globin genes bound HMG 17 to same extent, with 2-fold (or greater) enrichment over those containing non-erythroid specific genes. Erythroid cell derived core mononucleosomes containing the ϵ -globin gene

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bound HMG 17 at a level of enrichment lower than that displayed by those containing the ρ -globin and β -globin genes. Thymus cell-derived core mononucleosomes that contain genes of β -like globin gene cluster bound HMG 17 at a level comparable to non-erythroid specific genes in red blood cells. These results demonstrate the HMG 17 binding is tissue-specific, but not confined to the transcribed portions of active gene domains, and that preferential HMG 17 binding, alone, cannot account for the DNaseI-sensitivity of chromatin within the β -globin gene domain.

MATERIALS AND METHODS

Red blood cells were obtained from day 5 or day 14 White Leghorn chicken embryos or from 1.8 to 2 kg anemic adult White Leghorn chickens by phlebotomy. Adult chickens were made anemic by treatment with 1-acetyl-2-phenylhydrazine at 20 mg/kg for 4 or 5 days. Reticulocyte counts were above 90% at phlebotomy. 5-Azacytidine and sodium butyrate were injected intramuscularly as previously described (16). Thymus nuclei were prepared from frozen glands (Pel-Freez). Preparation of stripped, trimmed core mononucleosomes (referred to as mononucleosomes in the remainder of the paper) was as described by Brotherton and Ginder (3). Non-core histone protein contamination of mononucleosome preparations was less than 1%, as determined by SDS gel electrophoresis. HMG 17 was made from adult erythrocytes by the method of Bhullar and Candido (18). The purity of HMG preparations was ascertained by SDS-gel electrophoresis (3). Mononucleosomes were incubated at room temperature with HMG 17 in $2 \times$ TBE (0.18 M Tris, 0.18 M boric acid, 5 mM EDTA) for 5 min. prior to separation of HMG 17-bound from unbound mononucleosomes by electrophoresis in 5% acrylamide gels in $2 \times$ TBE (3).

The methods for DNA preparation and hybridization used in our laboratory have been described (3). Cloned DNA probes for β -globin (fkk36), ρ -globin (pJRho), ovalbumin (pOV-1) and a nontranscribed repeat sequence found in the ϵ -globin intervening sequence (ϵ -650) have been described previously (see Fig. 1) (3).

A unique probe for the ϵ -globin gene intervening sequence (ϵ -IVS) was made from the 1.8kb *Bam*-*Eco*R1 fragment of pCA β G1 by cutting with *Hpa* II to obtain a 550 bp fragment (13). A probe for the region immediately 5'-upstream to the β -globin cap site was made from the 2.1kb *Eco*R1-*Hind* III fragment of pCA β G1 by cutting with *Msp*I to obtain a 500 bp fragment (16). A unique IVS sequence of ρ -globin was the 400 bp *Sau*3A fragment of the 1.1 kb *Ava*I fragment of ρ b2H2 (16). The probe for thymidine kinase (*cTK*) is as described (19). A probe for malic enzyme was kindly provided by Dr. A. Goodridge (Dept. Biochemistry, Univ. of Iowa), and is described elsewhere (20). The determination of the ratio (R) of a given DNA sequence in the HMG 17-bound mononucleosome band to total DNA in this band was as follows. Bands in ethidium bromide stained gel photograph negatives and autoradiograms were scanned by laser densitometry (Biomed Instruments) and areas under curves (AUC) integrated. The value of R for a single lane was: $R = [(P_B/P_U)/(E_B/E_U)]$, where P is the AUC for bands on autoradiograms generated by hybridization of specific radiolabelled probes to HMG 17 bound (B) and unbound (U) mononucleosome DNA, and E is the AUC for bands in ethidium bromide stained gels. The mean R was determined from HMG 17 titrations in which between 15% and 45% of total mononucleosomes were HMG 17-bound, using the optimal lane in each titration experiment. Note that several exposures of each autoradiogram were obtained, and the exposure with band densities in the linear range of detection was selected for densitometric analysis. The variation in relative band intensity between exposures was low, typically less than 5%. The results of three separate titrations of adult and day 5 embryonic reticulocyte mononucleosomes and two titrations of day 14 embryonic reticulocyte mononucleosomes were used to determine the mean. This calculation seems justified as the variation for calculated R values between HMG 17 titrations in a single experiment was low when less than 50% of total mononucleosomes were HMG 17-bound. For example, for the gel shown in Fig. 2, the mean R ($\pm$ S.E.) for lanes 2 to 5 are, for fkk36, 1.9 ± 0.05 ; for ϵ -IVS, 1.2 ± 0.03 ; and for the

Figure 1: Map of the avian β -globin gene cluster. The position of the two embryonic globin genes, ρ and ϵ ; the two adult globin genes β^H and β ; and the cloned DNA probes used in this study are shown. The region of the erythroid specific enhancer element is marked by a stippled bar. Note that fkk22 is 5'- to the 5'-DNaseI hypersensitive region that is nucleosome free.

nontranscribed repeat sequence, 1.0 ± 0.06 . The results for thymus mononucleosomes are derived from a single titration experiment done in triplicate, with three lanes suitable for analysis. The photographs of autoradiograms shown in Fig. 2 and 3 are representative, and were chosen so that all bands were visible in the final figures.

RESULTS

HMG 17 has been reported to bind to the active chromatin of vitellogenin, lysozyme and ovalbumin genes only downstream from the respective starting points of transcription, and not in

Figure 2: HMG 17 binding to mononucleosomes from adult avian reticulocytes. Stripped core mononucleosomes (15 μ g/lane) were prepared from reticulocytes of anemic birds (lanes 1 to 5), and anemic birds treated with 5-azacytidine (lanes 6 to 9) or 5-azacytidine and sodium butyrate (lanes 10 to 14), and titrated with HMG 17. The ethidium bromide stained gel is shown in the top panel (a). DNA, transferred to nylon, was hybridized to probes for unique sequences in the β -like globin gene domain. Autoradiograms shown are for the: (b) adult β -globin gene (fkk36), (c) the embryonic ϵ -globin gene (ϵ -IVS), and, (d) the repeat sequence found in the ϵ -globin second intron. HMG 17 (1 mg/ml) added is: 0 μ l, lanes 1, 6, 10; 1 μ l, lanes 2, 7, 11; 2 μ l, lanes 3, 8, 12; 3 μ l, lanes 4, 9, 13; 4 μ l, lanes 5, 14. H, HMG 17-bound mononucleosomes; U, unbound mononucleosomes.

non-transcribed 5'-upstream regions (8). To determine whether a similar pattern of binding occurs in the β -globin gene cluster, we studied HMG 17-binding to mononucleosomes prepared from adult reticulocytes using a variety of probes (Fig. 2; Table 1). Autoradiograms generated from hybridization with the probe for the transcribed region of adult β -globin gene (Fig. 2b) were nearly identical to those obtained using probes for a region 200 bp upstream to the transcription initiation site of adult β -globin (fkk22) and the non-transcribed embryonic ρ -globin gene (pJRho). HMG 17 binding to these regions of chromatin was preferential over binding to bulk mononucleosomes (Fig. 2a) and to mononucleosomes that contained the embryonic ϵ -globin gene (ϵ -IVS) (Fig. 2c) or a nontranscribed repeat sequence (Fig. 2d). The degree of binding preference can be expressed by the ratio (R) of the gene probed to total DNA in the HMG 17-bound band. R averaged 1.9 to 2 for fkk36, fkk22, and pJRho (Table 1). In contrast, binding to mononucleosomes containing the ϵ -globin gene was intermediate ($R = 1.2$) between that found for other regions of DNA in the β globin cluster and for inactive DNaseI-insensitive genes such as ovalbumin and malic enzyme ($R = 1.0$) (Table 1). It is important to note that the preferential binding of HMG 17 to mononucleosomes containing both ρ -globin and β -globin was not due to cross-hybridization as identical results were obtained using a highly-specific probe for the second intervening sequence of ρ -globin (see Methods). Furthermore, we have previously shown that the ρ -globin probe used has no significant cross hybridization with β -globin under our reaction conditions (16).

To determine if transcriptional activity increases HMG 17-binding to mononucleosomes containing ρ -globin, anemic birds were treated with 5-azacytidine and butyrate. Exposure to 5-azacytidine results in demethylation of developmentally-regulated CpG sites 5'-upstream from ρ -globin, and expression of a developmentally-specific DNaseI-hypersensitive site in the previously described 5'-regulatory region of this gene (16). A high level of transcription of ρ -globin (up to 40% of the level of β -globin) does not occur unless the birds are also treated with butyrate. Although CpG sites in the ϵ -globin gene become demethylated, no detectable transcription occurs even after treatment with butyrate (16,17). As shown in Fig. 2 and Table 1, treatment with 5-azacytidine alone or with butyrate had no significant effect on HMG 17-binding to mononucleosomes from the β -globin cluster. We conclude that in the β -globin gene cluster, HMG 17 binds preferentially to both transcriptionally active and inactive regions of the domain. In addition, HMG 17-binding to adult reticulocyte chromatin mirrors transcriptional competence in the sense that chromatin containing the ρ -globin gene, which can be activated by treatment with 5-azacytidine and

Table 1: HMG 17-Binding to Mononucleosomes from Adult Avian Reticulocytes of Anemic Birds and Birds Treated With 5-Azacytidine and Butyrate

Ratio of Probed DNA to Total DNA in HMG 17-Bound Mononucleosomes (R)					
Probe:	pJRho	fkk22	fkk36	ϵ -IVS	Inactive
Treatment:					
control	1.9 ± 0.13	1.9 ± 0.38	2.0 ± 0.12	1.2 ± 0.04	1.0 ± 0.01
5-Azacytidine	1.8 ± 0.16	1.9 ± 0.14	1.9 ± 0.07	1.4 ± 0.07	0.9 ± 0.17
+ Butyrate					

Probes used are described in Methods. Inactive gene probes included ovalbumin (pOV₁), malic enzyme, and a nontranscribed repeat sequence found in the ϵ -globin second intervening sequence. Results represent the average of 3 or more separate experiments ($\pm$ S.E.), except for those shown for fkk22, for which only two experiments were done.

Figure 3: HMG 17 binding to mononucleosomes from primitive and definitive red blood cells of chick embryos. Stripped core mononucleosomes were prepared from primitive red cells of day 5 embryos (lanes 1 to 4, 10 μ g/lane), or from definitive red cells of day 14 embryos (lanes 5 to 8, 17 μ g/lane), and titrated with HMG 17. The ethidium bromide stained gel is shown in the top panel (a). DNA, transferred to nylon, was hybridized to probes for sequences in the β -like globin gene domain. Autoradiograms shown are for: (b) the embryonic ρ -globin gene (pJRho), (c) the adult β -globin gene (fkk36), (d) the unique sequences in the embryonic ϵ -globin gene (ϵ -IVS), and, (e) the repeat sequence found in the second intron of ϵ -globin. HMG 17 added is: 0 μ l, lanes 1, 5; 2 μ l, lane 2; 3 μ l, lanes 3, 6; 4 μ l, lanes 4, 7; 5 μ l, lane 8. H, HMG 17-bound mononucleosomes; U, unbound mononucleosomes.

butyrate, binds HMG 17 before and after activation as avidly as chromatin containing the β -globin gene, whereas chromatin containing the ϵ -globin gene binds HMG 17 relatively poorly and transcription is not activated by these treatments.

To examine further the relationship between HMG 17-binding and transcriptional competence, and to determine whether HMG 17-binding correlates with DNaseI-sensitivity, we studied HMG 17 binding using mononucleosomes prepared from day 5 and day 14 chick embryos. These cells were used for two reasons. First, embryonic red cells are metabolically more active than adult cells and therefore transcribe globin mRNA at a much higher rate (10,12,13). If ongoing transcription has an important effect on HMG 17-monomucleosome interactions, the higher rate of transcription in the embryonic cells should facilitate detection of differences in HMG 17 binding. Second, we have found it technically difficult to perform reliable DNaseI-titration studies in adult reticulocytes and thus we remain unsure as to whether the ϵ -globin gene is indeed sensitive to digestion in the adult cells.

Mononucleosomes from day 5 and day 14 red cells were titrated with HMG 17, as described above (see Materials and Methods) (3). As shown in Fig. 3, preferential binding of HMG 17 was detectable at the lowest HMG 17-to-monomucleosome ratios tested. The greatest degree of preferential binding was normally found when 10 to 20% of total mononucleosomes were bound. In contrast, with mononucleosomes from adult reticulocytes, preferential binding was not detected until 30 to 50% of total mononucleosomes were bound. Examination of the DNA sequences contained in the HMG 17-bound and unbound mononucleosomes shows preferential binding to those containing ρ -globin and β -globin ($R = 1.9$ to 2.5) (Fig. 3b and 3c), with intermediate binding to those containing ϵ -globin ($R = 1.3$ to 1.6) (Fig. 3d) when using mononucleosomes prepared from either day 5 primitive or day 14 definitive stage red cells (Table 2). Thus, the relative affinity of HMG 17 for mononucleosomes in the β -like globin gene cluster is similar in all red cell populations examined.

To ascertain that HMG 17 binding to mononucleosomes containing the β -globin gene cluster is tissue-specific, experiments similar to those shown above using fetal and adult reticulocytes were done using adult thymus gland nuclei. As shown in Table 2, HMG 17 did not bind preferentially to mononucleosomes containing the genes from the β -globin cluster. This was not due to a total absence of preferential binding by HMG 17 to mononucleosomes in the thymus cell preparations, as preferential binding to mononucleosomes containing the thymidine kinase (TK) gene was found ($R = 1.5 \pm 0.04$). The ratio of the binding of HMG 17 to mononucleosomes containing the TK gene relative to HMG 17-binding those containing either the ρ - or β -globin genes was about 2-fold (Table 2). Thymidine kinase is a housekeeping gene, and cell-cycle regulated (19).

Table 2: HMG 17-Binding to Mononucleosomes from Adult Thymus Cells, and Day 5 Primitive and Day 14 Definitive Chick Embryo Red Cells.

	Ratio of Probed DNA to Total DNA in HMG 17-Bound Mononucleosomes (R)			
	ρ -globin (pJRho)	β -globin (fkk36)	ϵ -globin (ϵ -IVS)	Non-transcribed Repeat Sequences
Day 5 RBC	1.9 ± 0.05	2.1 ± 0.58	1.3 ± 0.08	0.4 ± 0.04
Day 14 RBC	2.3 ± 0.49	2.4 ± 0.49	1.6 ± 0.11	1.0 ± 0.07
Thymus	0.7 ± 0.17	0.8 ± 0.16	0.5 ± 0.20	0.7 ± 0.17

Probes used are described in Methods. Results represent the average of 3 experiments ($\pm$ S.E.) for day 5 red cells, 2 experiments ($\pm$ S.E.) for day 14 red cells, and 1 experiment with 3 titrations for thymus cells.

Mononucleosomes that contain this gene are in chromatin that is DNaseI-sensitive and transcribed in adult reticulocytes (21), and preferentially bind HMG 17 in both adult, and day 5 and 14 embryonic reticulocyte preparations (data not shown). These results indicate that the preferential binding of HMG 17 to mononucleosomes containing the β -globin and ρ -globin genes is tissue-specific.

DISCUSSION

We have examined the binding of HMG 17 to mononucleosomes obtained from embryonic chicken reticulocytes of both primitive and definitive stages, from reticulocytes of anemic adult chickens treated with 5-azacytidine and butyrate, and from adult thymus cells. The principal finding of this paper is that erythroid cell mononucleosomes that contain sequences within or between the adult β -globin gene and the embryonic ρ -globin gene bind HMG 17 with ≥ 2 -fold enrichment over both bulk mononucleosomes and mononucleosomes containing genes that are inactive in erythroid cells. In contrast, the binding of HMG 17 to erythroid cell mononucleosomes containing the embryonic ϵ -globin gene is significantly decreased compared to this 2-fold enrichment; although the embryonic ϵ -globin gene, like the ρ -globin gene, is transcribed in primitive stage reticulocytes, and is DNaseI-sensitive in both primitive and definitive stage erythrocytes (11,13,15). No preferential binding to mononucleosomes containing β - or ρ -globin genes was detected when mononucleosomes were derived from adult thymus cell nuclei. The pattern of HMG 17 binding to erythroid cell mononucleosomes does not directly correlate with either transcriptional activity or the reported DNaseI-sensitivity of this domain; instead, it appears to correlate most closely with the potential for transcription within this gene cluster. As we have previously shown that preferential binding is not due to DNA length, single-strand nicks or bulk cytosine modification by methylation in the trimmed core mononucleosomes used in our studies (3), it is likely that preferential binding is conferred upon mononucleosomes by features intrinsic to the core histones. Possible features include variant histones (22,23) and post-translational histone modifications such as acetylation (24,25).

It is interesting to note that the ϵ -globin gene, in addition to binding HMG 17 poorly *in vitro*, is transcribed at low levels compared to the ρ -globin gene in primitive red blood cells (11,13). This low level of transcription occurs despite the fact that the ϵ -globin gene promoter can direct transcription as efficiently as the promoter of the β -globin gene, when assayed by transient transfection in either primitive or definitive avian red blood cells (26,27). It has been previously concluded that features other than *cis*-regulatory elements must effect ϵ -globin transcription (26). The studies presented in this paper suggest that the binding of HMG 17 may be one such feature, though the mechanism by which HMG 17 binding may facilitate transcription remains unclear.

Dorbic and Wittig (8) have reported on studies in several tissue-specific avian genes which show a transition in preferential HMG 17 binding, that corresponds to transcription initiation sites. These authors have concluded that HMG 17 binding must be confined to regions of active transcription, but this conclusion is not consistent with our results on the binding of HMG 17 to isolated core mononucleosomes from the β -globin gene cluster. One explanation for this discrepancy may be that HMG 17 proteins rearrange during chromatin isolation (28), and display preferential binding to longer mononucleosomes (3,29), and polynucleosomes

(7). Thus, chromatin from regions immediately 5'-upstream to transcription initiation sites, which often contain nuclease hypersensitive sites (30), may appear to be depleted of HMG 17 protein due to increased nuclease digestion in these regions in the course of preparing polynucleosomes for study. An alternative explanation may be that the transitions in HMG 17 binding detected at the 3'-end of the adult β -globin gene in the studies reported here and in those reported by Dorbic and Wittig are due to transitions in chromatin structure within intact nuclei that are not readily detected in isolated polynucleosomes or mononucleosomes. One such feature of chromatin in nuclei is that it is associated with the nuclear matrix. Transcription has been shown to occur at the interface between chromatin and the nuclear matrix (31), and it has previously been hypothesized that one function of HMG 17 may be to mediate the interaction between active gene domains and the nuclear matrix (32). This hypothesis was based on the observations that HMG 14 and 17, and the related protein HMG I may be associated with the nuclear matrix (32,33,34). In addition, HMG 14 and 17 have been shown to enhance the interaction of DNA with topoisomerase II (35), a major constituent of the nuclear matrix (36,37). In the ovalbumin gene, one of those examined by Dorbic and Wittig (8), nuclear matrix attachment has been shown to be confined to actively transcribed gene sequences in oviduct nuclei (38). We have recently mapped one tissue-specific nuclear matrix attachment region to a 480 bp *Hha* I-fragment 3' to the adult β -globin in both primitive and definitive avian red cells (39,40, and manuscript submitted). This same region contains the major erythroid-specific enhancer (Fig. 1) (26,27). The correlation between a change in HMG 17-binding to mononucleosomes and the position of a matrix attachment region both at the 3'-end of the β -globin gene, and of HMG 17-binding (8) and nuclear matrix association in the ovalbumin gene (38), support the hypothesis that HMG 17-binding to mononucleosomes may mediate the interaction between active gene domains and the nuclear matrix in an as yet undefined manner.

In conclusion, our studies on HMG 17 binding to chromatin from the β -globin gene domain of primitive and definitive erythrocytes suggest that a simple correlation between HMG 17 binding and transcription, and sensitivity to digestion by DNaseI, in this active gene domain is not present. Rather, it appears that HMG 17 binds *in vitro* to mononucleosomes that have the potential to display high levels of transcription *in vivo*. Further work is necessary to establish whether HMG 17 binding does indeed facilitate either the association of active chromatin with the nuclear matrix or transcription in intact nuclei. The avian β -globin gene cluster should provide a suitable model system for the further investigation of these questions.

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