The Architectural Transcription Factor High Mobility Group I(Y) Participates in Photoreceptor-Specific Gene Expression

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The nonhistone chromosomal proteins high mobility group I(Y) [HMG I(Y)] have been shown to function as architectural transcription factors facilitating enhanceosome formation on a variety of mammalian promoters. Specifically, they have been shown to act as a "molecular glue" mediating protein–protein and protein–DNA contacts within the enhanceosome complex. HMG I(Y) proteins are expressed at high levels in embryonic and transformed cells and have been implicated in transcriptional regulation in these cells. Terminally differentiated cells, however, have been reported to express only minimal, if any, HMG I(Y). In contrast to these observations, we show here that adult mouse retinal photoreceptors, which are terminally differentiated cells, express high levels of these proteins. Using retinoblastoma cells as an approximate model, we further demonstrate in transiently transfected cells that inhibition of HMG I(Y) expression and mutation of HMG I(Y) binding sites significantly reduce rhodopsin promoter activity. DNase I footprint analysis indicates that HMG I protein interacts with a discrete site within the rhodopsin proximal promoter. This site overlaps with the binding site for Crx, a paired-like homeodomain transcription factor that is essential for photoreceptor functioning and that when mutated causes several forms of human photoreceptor degeneration. Both biochemical and functional experiments demonstrate that HMG I(Y) physically associate with Crx and that their interaction with DNA is required for high-level transcription of the rhodopsin gene. These data provide the first demonstration that HMG I(Y) can be important for gene activation in terminally differentiated cells.

Key words: HMG I(Y); Crx; rhodopsin; retinoblastoma; retina; photoreceptors

Activation of eukaryotic gene expression relies on the formation of a multiprotein enhanceosome complex on promoters and enhancers adjacent to the transcription initiation site (Wolffe, 1994; Tjian and Maniatis, 1994; Grosschedl, 1995; Werner and Burley, 1997; Carey, 1998). Over the past few years, data indicated that the nonhistone chromosomal protein high mobility group I(Y) [HMG I(Y)] functions as a "molecular glue" in enhanceosomes (Thanos and Maniatis, 1995; John et al., 1996). This protein facilitates interaction of sequence-specific DNA-binding proteins to their target DNA sites and can act as a bridge between two DNA-binding proteins bound to nearby *cis* elements (Yie et al., 1997, 1999). To date, all examples of such a role for HMG I(Y) in gene activation have come from situations of either rapid cell division or "activation" (for review, see Bustin and Reeves, 1996; Bustin, 1999). Namely, HMG I(Y) and the highly related HMG I-C proteins have been implicated in enhanceosome formation during embryogenesis, lymphocyte activation (e.g., during lymphocyte proliferation and inflammation; for review, see Shannon et al., 1998), and tumorigenesis (for review, see Zhou and Chada, 1998; Tallini and Dal Cin, 1999). HMG I proteins are expressed most abundantly during embryogenesis (Chiappetta et al., 1996), lymphocyte activation (Himes et al., 1996, 2000; Lokuta et al., 1996; Khodadoust et al., 1999; Pellacani et al., 1999), and tumorigenesis (Giancotti et al., 1985, 1987, 1989; Ram et al., 1993; Tamimi et al., 1993; Chiappetta et al., 1995; Fedele et al., 1996; Bandiera et al., 1998; Giannini et al., 1999). This supports the view that these proteins are particularly important in these situations. In contrast, no HMG I-C gene expression is found in terminally differentiated cells. HMG I(Y) expression is either absent or occurs at 200-fold less than that the level observed during embryogenesis or during rapid cell division (Chiappetta et al., 1996).

Here we show that normal, terminally differentiated photoreceptor cells surprisingly express high levels of HMG I(Y) proteins. Because the transcription of photoreceptor-specific genes whose products are involved in phototransduction is a dynamic process controlled in a diurnal manner, we hypothesize that HMG I(Y) protein expression in these cells may be required to accommodate the daily induction of these genes. In support of this, we show using retinoblastoma cells as a model that HMG I(Y) expression is crucial for rhodopsin promoter activity, that HMG I proteins interact discretely in the proximal rhodopsin promoter overlapping with the BAT-1 *cis* element, and that mutagenesis of the HMG I(Y) binding site impairs promoter activity. Finally, we demonstrate that the paired-like homeodomain protein Crx, which binds to the BAT-1 site, physically interacts with HMG I(Y) and specifically with their DNA-binding domains. This interaction is reminiscent of interactions between HMG I(Y) proteins and the octamer family of POU-homeodomain proteins, which others and we have previously shown to facilitate the octamer factor binding and activity (Abdulkadir et al., 1995, 1998; Leger et al., 1995). In conclusion, this report provides the first evidence for an important role for HMG I(Y) proteins in gene activation in terminally differentiated cells and identifies an HMG-dependent gene in photoreceptor cells.

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MATERIALS AND METHODS

Preparation of ocular tissue and immunohistochemistry. Six- to 8-week-old BALB/c and C57BL/6 mice were obtained from the mouse colony at the Schepens Eye Research Institute, Harvard Medical School. Six-week-old A/J mice were obtained from Jackson Farms (Bar Harbor, ME). The mice were killed under ambient light in the mouse facility. The eyes were enucleated, placed immediately into Tissue-Tek OCT freezing media (Sakura, Torrance, CA), and frozen on dry ice.

Frozen sections (6 μm) of OCT-fixed samples were incubated in a solution of methanol and 1% hydrogen peroxide for 30 min before immunoperoxidase staining. After blocking overnight at 4°C with goat serum (1:33 dilution), slides were incubated with primary antibodies [rabbit anti-HMG I(Y) polyclonal antibodies; Chiappetta et al., 1995] (1:2000 dilution in PBS and goat serum) for 1 hr at room temperature. The slides were washed for 10 min in PBS containing 0.05% Tween 20 followed by two washes for 10 min each in PBS. Subsequently the slides were incubated with biotinylated goat anti-rabbit IgG for 1 hr (Vectastain Elite ABC kits; Vector Laboratories, Burlingame, CA). Staining was performed using DAB substrate (Vector, Burlingame, CA).

Cell culture. The retinoblastoma cells Y79 and WERI-Rb1 were maintained in Ham's F-12 and RPMI 1640 media (Life Technologies, Gaithersburg, MD), respectively, supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 50 μg/ml penicillin, 50 U/ml streptomycin (Sigma, St. Louis, MO), and 2 mM glutaMaxI (Gibco BRL). The cells were grown at 37°C in a humidified incubator with 95% air and 5% CO_2. Embryonic kidney 293 cells were cultured in DMEM (Life Technologies) with the same supplements.

Northern blot analysis. Twenty micrograms of total RNA isolated as described (Chomczynski and Sacchi, 1987) were resolved in 1% formaldehyde-denaturing gels and blotted to a Biodyne B nylon membrane (Pall, East Hills, NY). DNA probes [coding regions of mouse HMG Y (Johnson et al., 1988) and bovine Crx (Chen et al., 1997)] were radiolabeled with [^{32}P]dCTP (ICN, Costa Mesa, CA) by random priming (Mega-Prime DNA labeling kit; Amersham Pharmacia Biotech, Arlington Heights, IL) and purified by NucTrap push columns (Stratagene, La Jolla, CA). High-stringency hybridization in QuickHyb hybridization solution (Stratagene) and wash conditions were used. The blots were exposed to Fuji (Tokyo, Japan) Rx film with an intensifying screen (Sigma) at −70°C. Uniform RNA loading in individual sample was shown by hybridizing the same Northern blot with 28S ribosomal RNA probe.

Western blot analysis. Retinoblastoma Y79 and WERI-Rb1 cells were extracted with 5% perchloric acid (Giancotti et al., 1985), and equal amounts of the protein extracts were resolved by 22% Tricine-SDS-PAGE (Schagger and von Jagow, 1987) along with standards (Bio-Rad, Hercules, CA), transferred to Hybond-P (Amersham) as described (Patel et al., 1994), and probed with primary antibodies against HMG I(Y) as described (Chiappetta et al., 1995) and secondary antibodies coupled to alkaline phosphate for chemiluminescence detection (Western-Light; Tropix, Bedford, MA). The Western blot was prepared by equalizing amounts of perchloric acid extracts for HMG I proteins.

Transient transfection analysis. Retinoblastoma WERI-Rb1 cells were transfected by electroporation in 4 mm Cuvettes Plus cuvettes (BTX, San Diego, CA), in 300 μl of cell (3 million)-DNA mix in RPMI 1640 medium, at 1000 μF, infinite load resistance, 200 V, using the Electroporator II (Invitrogen, Carlsbad, CA), to deliver 20 or 50 μg of empty expression vector, or the cloned antisense HMG I(Y) (Thanos and Maniatis, 1992) or β-galactosidase (β-gal) gene at antisense orientation. Five micrograms of pbRho-130 reporter plasmid (Kumar et al., 1996) and 10 μg of pCAT-Control plasmid (to control for transfection efficiency; Promega, Madison, WI) were also co-transfected. Cells transfected were grown in 6 ml of completed RPMI 1640 medium. Forty hours later cells were harvested and assayed for luciferase activity using the Luciferase Assay System (Promega) and measured using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). The chloramphenicol acetyltransferase (CAT) activity was determined by CAT ELISA (Roche, Indianapolis, IN) and used to normalize luciferase reading. All plasmids used for transfection were prepared by Qiagen (Chatsworth, CA) plasmid kit. WERI-Rb1 cells were also co-transfected with 2 μg of the plasmid expressing green fluorescent protein and 20 μg of either the empty, antisense β-gal or antisense I(Y) plasmid, grown for 40 hr, and spun onto microscopic slides (Shandon, Pittsburgh, PA). The cells were stained with the polyclonal antibodies specifically recognizing the HMG I(Y) proteins as described above and Texas Red-conjugated secondary antibodies (Vector). The expression level of HMG I(Y) was analyzed by fluorescence microscopy (Eclipse E800; Nikon, Melville NY) and the use of Scion Image software (Frederick, MD).

To transfect 10 μg of pbRho-130 or pbRho-130 mutHMG I (see below) reporter and 3 μg of pCAT-Control (Promega) plasmids into 30,000 WERI-Rb1 cells, TransFast (Promega) was used according to the supplier's instructions. Briefly, DNA was mixed with 26 μg of TransFast reagent and incubated, and then cells in RPMI 1640 medium were added. After 8 hr, 3 ml of completed RPMI 1640 medium was added, cells were grown, and luciferase and CAT activities were assayed and normalized as described above.

293 cells were transfected by LipofectAMINE (Life Technologies), according to the manufacturer's recommendation. In short, 0.3 μg of pbRho-130 or pbRho-130 mutHMG I reporter plasmid was mixed with bCrx expression vector (Chen et al., 1997) in steps of 0, 0.03, 0.06, and 0.3 μg and compensated by the empty vector so that 0.3 μg of expression vector and 1 μg of pCAT-Control plasmid (Promega) were always transfected. Five micrograms of LipofectAMINE reagent were added, and after incubation the whole mixture was added to 293 cells grown in 35 mm dishes at 50% confluence. Eight hours later 2 ml of completed DMEM was added, and after 40 hr cells were harvested and assayed for luciferase and CAT activities.

DNase I footprint analysis. The bovine rhodopsin proximal promoter region (bRPPR) from −130 to +70 bp was excised from plasmid pbRho-130 (Kumar et al., 1996) and radiolabeled, either the coding or noncoding strand, with ^{32}P (ICN) for analysis. DNase I footprinting procedures optimized for HMG I protein have been described (Abdulkadir et al., 1995). DNase I used was purchased from Promega (RQ1 DNase). Recombinant HMG I protein and fusion protein of glutathione *S*-transferase (GST) and Crx-homeodomain (GST-Crx-HD) were prepared as described (Arlotta et al., 1997; Chen et al., 1997). Total cell lysates of the retinoblastoma cells were prepared as described (Abdulkadir et al., 1995).

Electromobility shift assays and site-directed mutagenesis. The probe for the BAT-1 site was prepared from oligonucleotides annealed as a duplex, whose upper strand sequence is 5′-GTGAGGATTAATATGATTAATA-ACGCCCC-3′. The BAT-1 site with a mutated HMG I(Y) binding site (BAT-1 mutHMG I) has the sequence 5′-GTGAGGATTCCTATGATTC-CTAACGCCCC-3′; underlined are the nucleotide changes. Probes were radiolabeled with [^{32}P]ATP (ICN). Conditions for gel shift experiments of Crx (Chen et al., 1997) and HMG I protein (Abdulkadir et al., 1995) have been described.

The oligonucleotides of the BAT-1 site with the mutated I(Y) binding site were used for overlapping PCR to amplify a fragment of bRPPR from −130 to + 70 bp that carried mutations. The amplified fragment was cloned into the same expression vector as pbRho-130 (pcDNA 3.1/His C; Invitrogen). The sequence of the mutant plasmid pbRho-130 mutHMG I was confirmed by DNA sequencing (Tufts DNA Sequencing Facility, Physiology Department, Boston, MA).

GST pulldown assays. Immobilization of GST alone and GST-HMG I to glutathione-Sepharose beads (Pharmacia, Piscataway, NJ) has been described (Du et al., 1993). Radiolabeled bCrx or luciferase was prepared using the coupled *in vitro* transcription and translation kit (Promega) and [^{35}S]methionine (ICN) as instructed by the manufacturer. Beads were mixed with radiolabeled bCrx or luciferase in 25 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, and 0.25% BSA at 4°C for 2 hr. Then beads were washed two times with the same buffer and two times with buffer without BSA. Bead-bound proteins were eluted by boiling in SDS-PAGE loading buffer and analyzed by SDS-PAGE. After electrophoresis, the gel was stained with Coomassie blue, destained, dried, and exposed to x-ray film (Fuji Rx Film) for autoradiography.

Far-Western blot analysis. The GST-HMG I deletion proteins and the experimental procedure have been described previously (Yie et al., 1997; Abdulkadir et al., 1998). ^{35}S-Labeled bCrx was prepared as above for probe.

Data analysis. Values are presented as mean ± SE (SD divided by square root of n). Student's t test was conducted using two-sample assuming equal variances, and the p value considered significant was stated.

RESULTS

An unexpected expression of HMG I(Y) proteins in normal adult retina

Normal retinal function depends on the rapid turnover and synthesis of many proteins (e.g., rhodopsin, transducin, and retinol-binding protein) within photoreceptor cells (Albert and Jakobiec, 1994; Argamaso et al., 1995). In an effort to understand the molecular mechanisms that permit such high rates of gene expression, we analyzed the expression of the chromatin-associated HMG I(Y) proteins in these cells. Immunohistochemistry of ocular cryosections from two adult albino mouse strains, BALB/c and A/J, and a pigmented strain, C57BL/6, was performed using purified anti-HMG I(Y) polyclonal antibodies.

Unusually strong nuclear staining by anti-HMG I(Y) antibodies was observed in the cell bodies of photoreceptors (outer nuclear layer; Fig. 1*A*). The outer plexiform layer and the inner and outer segment regions of the photoreceptors were not stained. The nuclei of the ganglion cells, inner nuclear layer, and retinal pigment epithelium were weakly immunostained. Very low background immunoreactivity was observed in the nuclei of muscle cells attached to the eye (extraocular muscle). Staining from endogenous enzyme was not seen in the control sections in the absence of primary antibodies (data not shown). Preabsorption with the immunogenic peptide substantially reduced staining, demonstrating the specificity of the antibodies (data not shown). Northern blot analysis of RNAs isolated from retina, skeletal muscle, heart, and

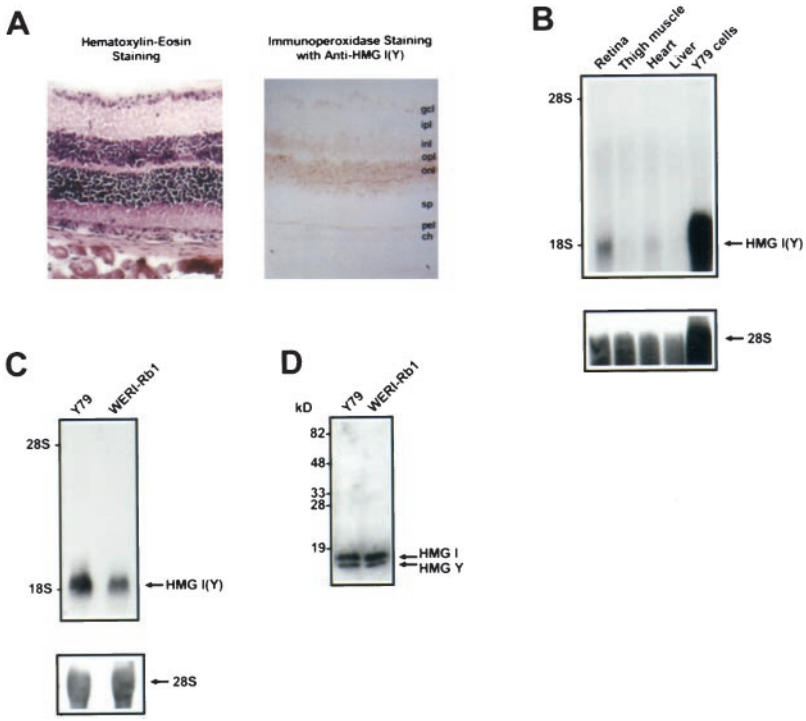

Figure 1. High-level expression of HMG I(Y) in nuclei of photoreceptor cell bodies in normal adult mouse retina and in retinoblastoma cells. *A*, Photomicrograph of a representative longitudinal cryosection of a BALB/c eye stained by hematoxylin-eosin (*left panel*) and immunoperoxidase (*right panel*) and the polyclonal antibodies against HMG I(Y). See Results for detailed description. The same results were obtained from at least five staining experiments and from that of A/J and C57BL/6 mice (results not shown). Magnification, 400×. *ch*, Choroid; *gcl*, ganglion cell layer; *inl*, inner nuclear layer; *ipl*, inner plexiform layer; *onl*, outer nuclear layer; *opl*, outer plexiform layer; *pel*, pigmented epithelium layer; *sp*, segments of photoreceptors. *B*, Northern blot analysis of intact total RNA prepared from retina, thigh muscle, heart, and liver of BALB/c mice and Y79 retinoblastoma cells to detect HMG I(Y) transcripts (*top panel*). HMG I(Y) mRNA is expressed at a higher level in retina than that in other examined mouse tissues. *Bottom panel,* Equal RNA loading was shown by probing the same blot with 28S rRNA, except that Y79 cells were included in the blot as control and marker, although Y79 RNA was overloaded. *C*, Total RNA isolated from retinoblastoma Y79 and WERI-Rb1 cells was analyzed for HMG I(Y) transcripts by Northern blotting. This demonstrates the expression of HMG I(Y) RNAs in retinoblastoma cells. As shown in the *top panel*, the two alternatively spliced transcripts of HMG I(Y) are ~2 kb in size (Johnson et al., 1988). The 28S bands shown in the *bottom panel* indicate the integrity of RNA loaded. *D*, Western blot analysis of HMG I and HMG Y proteins in retinoblastoma Y79 and WERI-Rb1 cells. Results show that HMG I(Y) proteins are present in retinoblastoma cells. HMG I and HMG Y are 11.9 and 10.6 kDa, respectively (Johnson et al., 1988).

liver (Fig. 1*B*) demonstrated that adult, normal mouse retina expressed markedly elevated levels of HMG I(Y) transcripts. The high level of HMG I(Y) expression observed in the Northern analysis is in agreement with the immunohistochemical analysis shown in Figure 1*A*.

HMG I(Y) expression in retinoblastoma cells

The previous experiments established that normal adult photoreceptor cells express unusually high levels of HMG I(Y) proteins. Because these proteins have been shown to function as facilitators of enhanceosome formation, we hypothesize that their presence in photoreceptor cells at high levels may signify that they are important for photoreceptor-specific, inducible gene expression. Indeed, photoreceptor cells undergo daily cycles of gene expression (Brann and Cohen, 1987; Bowes et al., 1988). For example, the rhodopsin gene appears to be under diurnal control, with rhodopsin synthesis initiating before light onset and decreasing before light offset (Korenbrot and Fernald, 1989; Farber et al., 1991).

Because retinoblastoma cells have been used as an approximate model to study photoreceptor gene expression (Di Polo and Farber, 1995; Fong and Fong, 1999), we analyzed these cells to see whether they resemble photoreceptor cells with respect to HMG I(Y) expression. As can be seen in Figure 1, *B* and *C*, both Y79 and WERI-Rb1 retinoblastoma cells express high levels of HMG I(Y) transcripts. In addition, both of these cells express both HMG I and HMG Y proteins as determined by Western blot analysis (Fig. 1*D*). We therefore decided to use these cells in elucidating the role of HMG I(Y) in photoreceptor function.

Reduction of rhodopsin promoter activity by antisense HMG I(Y) RNA expression

We have focused our attention on the rhodopsin gene as a potential HMG I(Y)-dependent gene, because its expression is diurnal and its gene promoter is among the best characterized of the photoreceptor-specific genes (Nathans, 1987; Kumar and Zack, 1994). We first demonstrated that the WERI-Rb1 retinoblastoma cell line supports rhodopsin promoter activity as assessed using transient transfection with a luciferase reporter assay (data not shown). Antisense HMG I(Y) RNA was expressed in this cell line to determine whether HMG I(Y) is required for rhodopsin gene expression. We and others have previously obtained evidence that HMG I(Y) participates in constitutive and/or inducible gene expression via inhibition of this expression using antisense HMG I(Y) RNA (Thanos and Maniatis, 1992; Abdulkadir et al., 1995; Himes et al., 2000; Scala et al., 2000). The data presented in Figure 2*A* show that rhodopsin promoter activity, specifically from the bRPPR −130 to +70 bp (Kumar et al., 1996) in the WERI-Rb1 cell line, is significantly inhibited by expression of antisense HMG I(Y) RNA versus co-transfection of equivalent amounts of empty vector or the same vector cloned with the β-galactosidase gene in antisense orientation (*white*, *gray bars,* respectively). Increasing the dose of antisense HMG I(Y) expression vector further reduces the rhodopsin promoter activity. This experiment provided us with the first evidence that HMG I(Y) expression is important for rhodopsin gene expression.

To confirm that expression of antisense HMG I(Y) RNA reduced the level of HMG I(Y) proteins in the transfected retinoblastoma cells, we co-transfected the cells with one of the effector plasmids [empty, anti-β-gal, or anti-I(Y)] and 1/10 the amount of a green fluorescent protein expression plasmid and determined the I(Y) protein level of the transfected cells by immunocytochemistry using the specific antibodies recognizing I(Y) proteins as described above and Texas Red-conjugated secondary antibodies. Only cells exhibiting green fluorescence were analyzed for red fluorescence signals, using the Scion Image software. Shown in Figure 2*B* is the mean fluorescence signals from the cells transfected with the effector plasmids, and SEs are indicated as error bars. Figure 2*C* shows an example of the fluorescent images. *Arrows* highlight two selected cells from each transfection, and they harbored the plasmid and therefore displayed green fluorescence. Red fluorescence [revealing HMG I(Y) protein level] was substantially lower in the cells expressing antisense I(Y) RNA (*bottom row*) when compared with that transfected with empty vector (*top row*) or antisense-β-galactosidase (*middle row*).

Recombinant HMG I protein footprints a discrete site in the rhodopsin proximal promoter

Because antisense HMG I(Y) RNA was shown to inhibit rhodopsin promoter activity specifically, we next set out to identify HMG I(Y) binding sites within the rhodopsin proximal promoter. Figure 3*A* shows the results of *in vitro* DNase I footprint experiments using recombinant HMG I protein. Both the coding and the noncoding strands of the −130 to +70 bp of bRPPR were footprinted in this

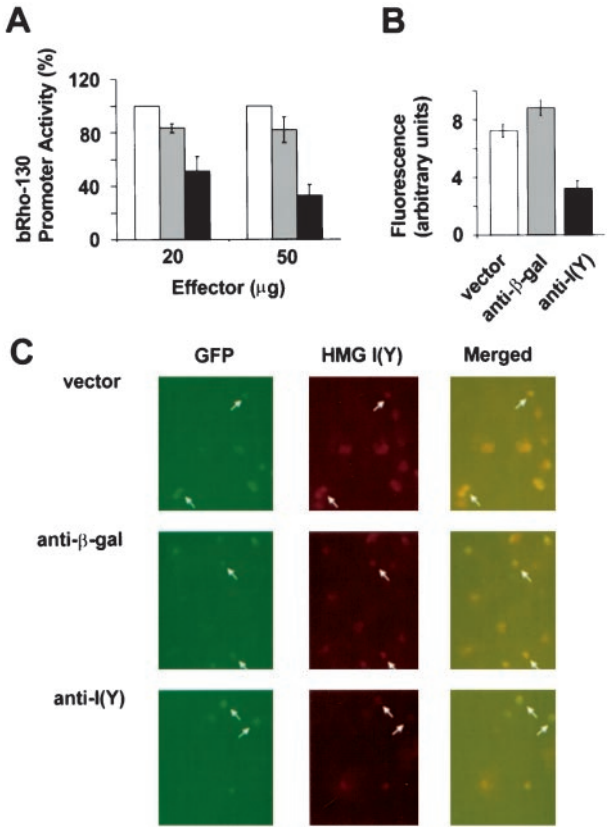

Figure 2. Blockade of HMG I(Y) expression in retinoblastoma cells reduces the promoter activity of the first 130 bp of bRPPR. *A*, Three million retinoblastoma WERI-Rb1 cells were transiently co-transfected with the pbRho-130 luciferase reporter plasmid and effectors of either the empty vector or the same vector cloned with the HMG I(Y) or β-galactosidase gene in antisense orientation, and the corresponding rhodopsin promoter activities co-transfected with the antisense β-galactosidase (*gray bars*) or antisense I(Y) (*black bars*) expression plasmid are shown as percentage decrease relative to that co-transfected with the empty vector (*white bars*). Results are summarized as means ± SE (error bars) of at least three independent experiments. A decrease (to 51%) in rhodopsin promoter activity was observed from transfection of the antisense I(Y) expression plasmid (20 μg) but not that from the other antisense plasmid transfection. A more substantial decrease in rhodopsin promoter activity (to 33%) was seen with a higher level of antisense HMG I(Y) expression plasmid (50 μg). The decrease in rhodopsin promoter activity by blocking HMG I(Y) expression was considered significant after the normalized luciferase readout (by co-transfecting a CAT expression plasmid and the luciferase activity normalized by the CAT activity) was evaluated using Student's *t* test, $p < 0.02$, and under this condition the difference of rhodopsin promoter activity between that co-transfecting the empty vector and the antisense β-galactosidase was deemed not significant. *B*, WERI-Rb1 cells transfected with 20 μg of an effector and 2 μg of a plasmid expressing green fluorescent protein (GFP) were cytospun onto microscopic slides and immunostained for HMG I(Y) proteins using procedures similar to those above, but immunofluorescence was detected with antibodies conjugated with Texas Red. Fluorescence microscopy was performed, and images were captured, with the same exposure time and sensitivity of detection, on four random fields of an individual slide (one transfection experiment), using filters of 507 nm (for GFP) and 596 nm (for Texas Red) for quantification. Red fluorescence signals were measured (on every cell within the field) using the Scion Image software only on those cells that displayed green fluorescence. Pooled red fluorescence signals from the examined four fields of individual transfection were shown as mean arbitrary fluorescence (i.e., 1.81, 2.20, and 0.81, respectively) and SE, in which *white*, *gray,* and *black bars* represent transfection of empty, anti-β-gal, and anti-I(Y) plasmid, respectively. In total, 130, 132, and 71 cells were analyzed from those transfected with empty, anti-β-gal, and anti-I(Y) plasmid, respectively. The experiment was repeated twice, and similar results were obtained. Student's *t* test determined that the variance of red fluorescence from anti-I(Y) transfection is significantly lower ($p < 0.000001$) when compared with that transfected with empty vector or anti-β-gal. *C*, Example of fluorescent microscopy images of transfected retinoblastoma cells described in *B*, showing in the *left column* green fluorescence exhibited by green fluorescent protein (*GFP*) expression, in the *middle column* red fluorescence displayed by HMG I(Y) immunostaining, and in the *right column* merged images (*yellow* indicates co-localization). *Arrows* highlight the selected cells of interest, from which red fluorescence exhibited from cells [revealing HMG I(Y) level] transfected with anti-I(Y) plasmid is weaker than that of the empty or anti-β-gal vector, and therefore the merged signal (with GFP) appears *greenish yellow* rather than *yellow*.

experiment. Footprint analysis on the coding strand demonstrates two tandem footprinted sites, which we have designated A and B. The same sites are protected in the noncoding strand footprint but appear as a single footprint. The nucleotide sequence of footprinted region A and B is shown in Figure 3*C*. Inspection of this footprinted site showed that the HMG I(Y) binding site coincides with the BAT-1 *cis* element in the rhodopsin gene with which the paired-like homeodomain protein Crx interacts (Chen et al., 1997; Furukawa et al., 1997).

Figure 3*B* illustrates how HMG I(Y) interact with the BAT-1 element (*lanes 10*, the coding and noncoding strands), whereas Crx alone interacts with both the BAT-1 and Ret 4 sites (*lanes 11*, both the coding and noncoding strands). Under conditions optimized for HMG I(Y) footprinting, patterns illustrated in *lanes 2–4* and *6–8* demonstrated that the shared HMG I(Y)–Crx binding site at BAT-1 strongly footprinted by extracts from the retinoblastoma Y79 and WERI-Rb1 cells are consistent with HMG I(Y) binding.

The HMG I(Y) binding site in BAT-1 is critical for rhodopsin promoter activity

With the aim of obtaining further evidence that HMG I(Y) interaction with the BAT-1 element is critical for rhodopsin promoter activity, we inspected the sequence specificity of Crx binding and made site-specific mutations in the BAT-1 site to selectively interfere with HMG I(Y) binding. Figure 3*C* illustrates the two Crx binding sites (BAT-1 and Ret 4, which are *bracketed*) and the HMG I(Y) binding site, designated A and B. Four A nucleotides were site specifically mutated to four C nucleotides, as shown on the *top*. Because HMG I(Y) usually requires in excess of four consecutive A or T nucleotides to bind to DNA (Reeves and Nissen, 1990; Arlotta et al., 1997), these substitutions were predicted to interfere with HMG I(Y) binding. In contrast, a previous study of the sequence specificity of Crx indicated that these changes should not affect Crx interaction with the BAT-1 site (Chen et al., 1997; Furukawa et al., 1997). The gel mobility shift assay shown in Figure 4*A* demonstrates that our site-specific mutations indeed selectively interfere with HMG I(Y) interaction with the BAT-1 site. The gel shift experiment in the Figure 4*A, left panel*, assesses the ability of the recombinant GST-Crx-homeodomain fusion protein to interact with the wild-type and site-specifically mutated BAT-1 elements. Increasing amounts of recombinant protein were used in *lanes 2–4* and *6–8*. No significant impact on Crx binding is observed when the mutated BAT-1 element is used as a probe. In contrast, the gel shift experiment in the Figure 4*A, right panel*, shows that the site-specific mutations in the BAT-1 element almost completely interfere with the ability of recombinant HMG I protein to interact with the site. The experiments in Figure 4*A* established that we had chosen appropriate mutations to assess the specific contribution of HMG I–protein interaction with the BAT-1 site in rhodopsin gene transcription.

We showed that the retinoblastoma cells support rhodopsin promoter activity and express high levels of HMG I(Y) protein. After discovering that HMG I(Y) proteins footprint the BAT-1 site overlapping to the Crx binding site, we determined whether these cells express the Crx transcription factor. A Northern blot is shown in Figure 4*B*, which demonstrates that the two retinoblastoma cells express three transcripts of the Crx gene (Freund et al., 1997). Therefore, this cell system (1) supports rhodopsin activity, (2) expresses high levels of HMG I(Y) proteins, and (3) expresses the Crx gene. We proceeded along to transfect both the wild type and mutant rhodopsin reporter constructs into two different cell systems to assess the importance of the HMG I(Y) binding site in rhodopsin promoter activity. In Figure 4*C*, the wild-type and HMG I(Y) site–mutant reporter constructs have been transfected into

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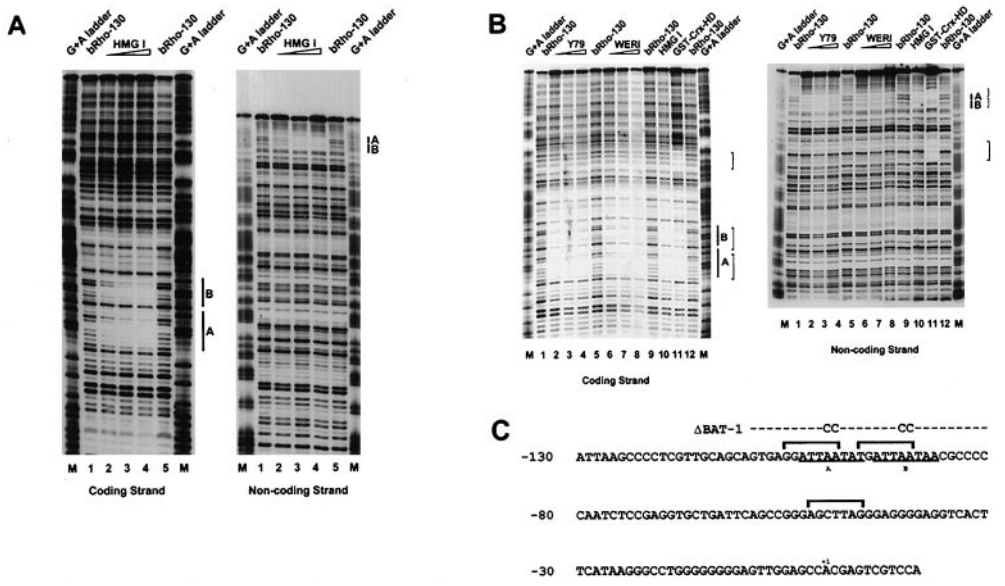

Figure 3. HMG I proteins bind to the bRPPR at the BAT-1 site. *A*, DNase I footprint analysis of bRPPR template from −130 to +70 bp with bacterially expressed HMG I protein. *Left panel*, bRPPR with the coding strand labeled; *right panel* bRPPR with the noncoding strand labeled. *Lane M*, G + A chemical cleavage sequencing reaction; *lanes 1* and *5,* no added protein; *lanes 2–4,* increasing amount of HMG I protein (i.e., 10, 100, and 500 ng). Two strong protected regions are identified, indicated as *A* and *B*. The same results were obtained after the experiment was repeated twice. *B*, HMG I(Y) from retinoblastoma cells bind to bRPPR, demonstrated by DNase I footprinting of the same bRho-130 region with whole-cell lysate of retinoblastoma Y79 and WERI-Rb1 cells. *Lane M,* G + A ladder; *lanes 1, 5, 9, 12*, no added protein; *lanes 2–4*, increasing level of Y79 cell lysate; *lanes 6–8*, increasing level of WERI-Rb1 cell lysate; *lanes 10, 11*, 100 ng of each bacterially expressed fusion protein of GST and Crx-homeodomain (GST-Crx-HD) and HMG I protein added, respectively. Binding sites for Crx are *bracketed*. *Left* and *right panels,* Radiolabeled coding and noncoding strands, respectively. The experiment was performed three times with similar results. *C*, Nucleotide sequence of the bRPPR from −130 to +70 bp. The A and B sequences are protected by HMG I overlap with the BAT-1 site. Regions protected by the recombinant Crx homeodomain are *bracketed*. Oligonucleotide (used for subsequent gel shift experiments) containing the BAT-1 site and the BAT-1 site with a mutation in the HMG I binding site are shown. Nucleotide *A* marked as *+1* is the transcription start site of the gene.

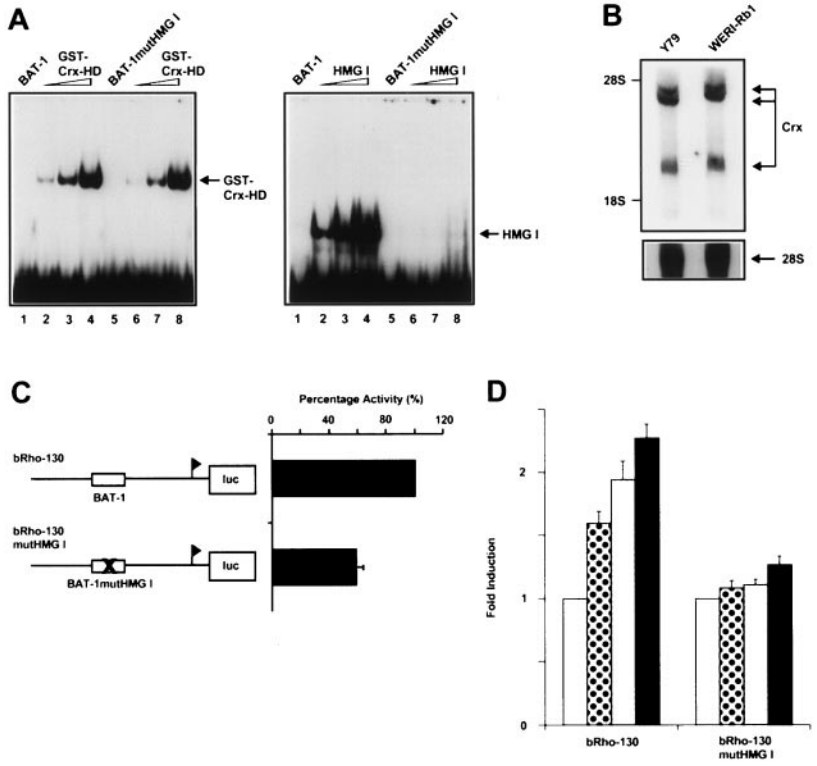

Figure 4. Binding of HMG I(Y) in the BAT-1 site contributes to the transactivation of Crx to the bovine rhodopsin proximal promoter. *A*, Demonstration of the BAT-1 site with the mutated HMG I(Y) binding site (*BAT-1mutHMG I*) prohibits HMG I binding but does not affect Crx binding. Gel shift experiments were performed with a bacterially expressed fusion protein of GST and Crx homeodomain (*GST-Crx-HD*; *left panel*) and HMG I (*right panel*). Radiolabeled oligonucleotides containing the wild-type BAT-1 site (*lanes 1–4*; BAT-1) and the BAT-1 with mutations of HMG I(Y) binding sites (Fig. 3*C, lanes 5–8*; BAT-1mutHMG I) were incubated with increasing level of GST-Crx-HD (0.4, 2, and 10 ng from *lanes 2–4, 6–8*; *lanes 1, 5* had no protein) and HMG I (4, 20, and 100 ng) and electrophoresed onto native polyacrylamide gels. Similar amounts of probe–Crx complexes are observed between wild-type and mutant probe. Essentially no mutant probe–HMG I complex was seen. Identical results were obtained in a repeated experiment. *B,* Northern blot analysis showing the expression of Crx in the two retinoblastoma cells, Y79 and WERI-Rb1. The sizes of Crx transcripts shown in the *top panel* are similar to those previously reported (Freund et al., 1997). *Bottom panel,* The total RNA on the same blot is intact and of similar loading after probing with 28S rRNA. *C*, Thirty thousand WERI-Rb1 retinoblastoma cells were transiently transfected with the pbRho-130 or pbRho-130 mutHMG I luciferase reporter and pCAT-Control plasmids (for normalization of transfection efficiency) and 40 hr later were harvested and assayed for luciferase and CAT activities. Promoter activity is shown relative to the luciferase activity normalized by the CAT activity of pbRho-130 with the BAT-1 site deleted. Results are expressed as means ± SE (error bars) of at least three independent experiments. Rhodopsin promoter possessing mutant HMG I(Y) binding sites at the BAT-1 site has weaker activity (59%) than that of the wild type in the context of retinoblastoma cells. Their difference is significant ($p < 0.00001$) by means of Student's t test for normalized luciferase reading. *D*, 293 cells were transiently co-transfected with the pbRho-130 or pbRho-130 mutHMG I reporter, the bovine Crx expression vector, and the pCAT-Control plasmid. Promoter activity of the reporter is shown relative to the normalized luciferase activity, monitored by CAT level, of the absence of bCrx expression vector (*white bars*). *Dotted, striped*, and *black bars* represent 0.03, 0.06, and 0.3 μg, respectively, of Crx expression vector transfected. Results are presented as means ± SE (error bars) of at least three independent experiments. Crx transactivates rhodopsin promoter to a greater extent (1.59-, 1.94-, and 2.27-fold induction, respectively; *left panel*) than that from its mutant (1.08-, 1.11-, and 1.26-fold induction, respectively; *right panel*) carrying disrupted HMG I(Y) binding sites at the BAT-1 site. Using Student's t test and $p < 0.0001$, the fold induction of rhodopsin promoter activity from wild-type reporter is considered statistically significant, but that from the mutant is not.

our WERI-Rb1 retinoblastoma cell system. Elimination of the HMG I(Y) binding site results in a marked decrease in rhodopsin promoter activity in this cell system. To provide additional evidence that the HMG I(Y) binding site is critical for transactivation of the rhodopsin promoter by Crx, we used a second cell system developed by D. J. Zack's group (Chen et al., 1997), where the 293 cells [which are HMG I(Y)-positive and Crx-negative; data not shown] were co-transfected with the wild-type or mutant rhodopsin reporter constructs and the Crx expression vector. Although Crx expression was able to transactivate the wild-type rhodopsin reporter construct in a dose-dependent manner, the rhodopsin reporter carrying the mutation in the HMG I(Y) site was nonrespon-

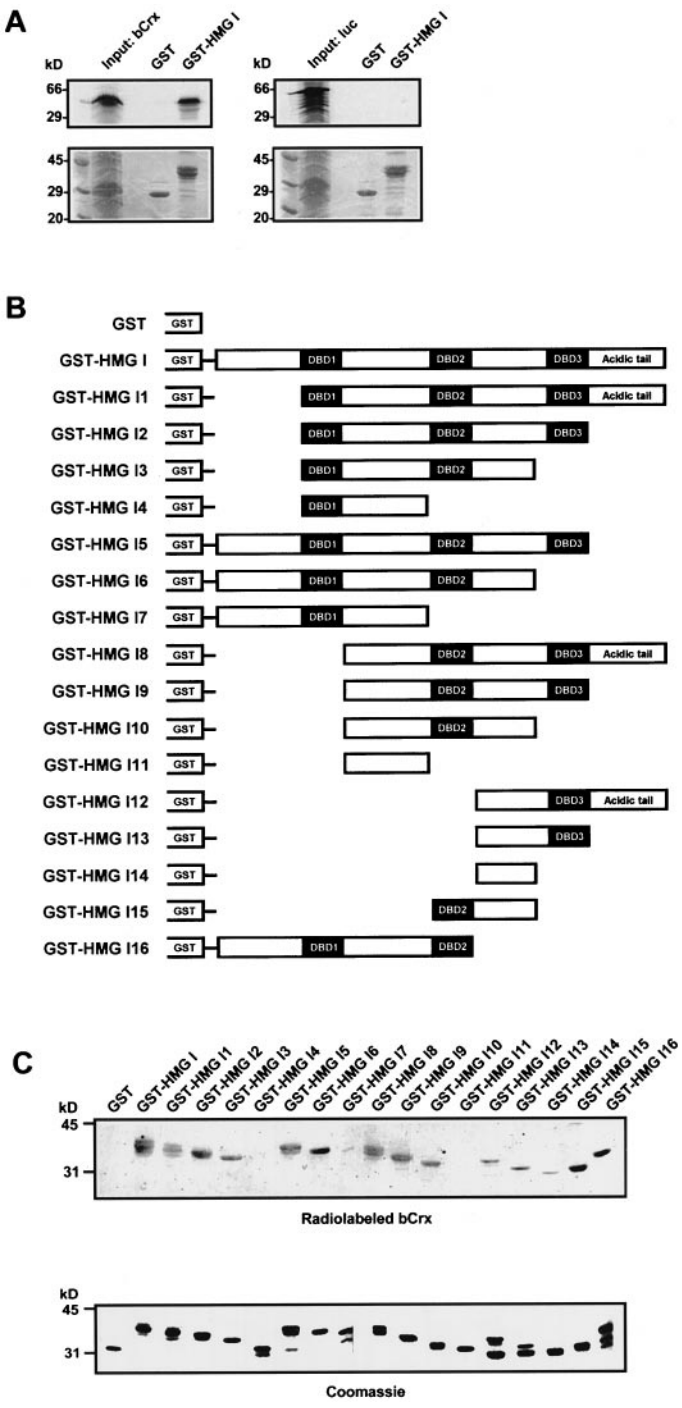

Figure 5. HMG I(Y) interacts with Crx. *A*, GST pulldown assays showing that HMG I(Y) physically interacts with Crx. GST alone and GST-HMG I immobilized on glutathione-Sepharose beads were incubated with bCrx (*left panel*) and luciferase (*right panel*; used as a negative control) translated with a rabbit reticulocyte lysate and labeled with [^{35}S]methionine. After extensive washing, binding was assessed by SDS-PAGE and autoradiography. The input lanes contain 50% of each ^{35}S-labeled protein used in the binding assays. *Bottom panels,* Same loading of immobilized GST and GST-HMG I. This experiment was performed in triplicate, and the results were identical. *B*, Schematic representation of the HMG I deletions used in the Far-Western blot analysis as described below. The three DNA-binding domains of HMG I are denoted as *DBD1–DBD3*. *C*, Far-Western blot analysis mapping HMG I(Y) interaction sites for Crx. Equal amounts (indicated in the *bottom panel*) of GST and GST-HMG I full-length and deleted proteins were resolved and transferred to a nitrocellulose membrane. The proteins on the blot were denatured, renatured, and then probed with ^{35}S-labeled translated bCrx. *Top panel*, Autoradiographed blot, in which no interaction occurs between HMG I mutants and Crx when both DBD2 and DBD3 are deleted (i.e., radiolabeled Crx did not bind to I4, I7, and I11 and only very weakly to I14, because no or very weak bands are seen in the corresponding lanes). Removing DBD1 does not have much effect on their interaction when comparing intensity of bands between I8–I11 and I1–I4. This suggests that HMG I(Y) specifically interact with Crx using DBD2 and DBD3. The same results were obtained from the experiment independently performed three times.

sive to Crx expression. Therefore, we have shown, using two different cell systems, that a competent HMG I(Y) interaction site in BAT-1 is critical for rhodopsin promoter activity and specifically for Crx transactivation.

Evidence for physical association between Crx and HMG I(Y)

The demonstration via experiments shown in Figure 4 that an intact HMG I(Y) binding site is critical for rhodopsin gene expression raised the issue of how HMG I(Y) proteins participate in the formation of an enhanceosome complex on this promoter. HMG I(Y) proteins have been previously shown to participate in enhanceosome formation on eukaryotic promoters by promoting protein–protein and protein–DNA interactions. In three previously characterized situations, HMG I(Y) proteins have bound to the specific *cis* elements, changed the local DNA conformation in allowing an easier access for the transcription factors, and physically interacted with the transcription factors in facilitating their loading onto the *cis* elements. This has been demonstrated for bZIP, Rel, and POU-homeodomain families of transcription factors (Du et al., 1993; Lewis et al., 1994; Whitley et al., 1994; Abdulkadir and Ono, 1995; Abdulkadir et al., 1995; Falvo et al., 1995; Thanos and Maniatis, 1995). HMG I(Y) binding to BAT-1 and interaction with Crx may participate in rhodopsin gene transcription in a similar manner.

To begin to probe this mechanism, we have initiated experiments to determine whether HMG I(Y) proteins and Crx physically interact. Using the GST pulldown assay, we tested the ability of recombinant HMG I bound to beads to pull down radiolabeled Crx and luciferase (as a negative control). As can be seen in Figure 5*A*, HMG I protein can physically associated with Crx but not the negative control luciferase. No Crx is pulled down with beads coupled to GST alone. These data demonstrate that HMG I(Y) proteins and Crx physically interact, as is the case with other POU-homeodomain proteins (Abdulkadir et al., 1995, 1998; Leger et al., 1995).

Next, we examined whether the interaction between HMG I(Y) proteins and Crx is specific and mapped the regions in HMG I(Y) responsible for such interaction. We and others have used Far-Western blot analysis in addressing similar issues (Yie et al., 1997; Abdulkadir et al., 1998). Data obtained from the experiment shown in Figure 5, *B* and *C*, suggests that the presence of at least DNA-binding domain 2 or 3 (DBD2 or DBD3) of HMG I is critical for its association with Crx, and that the rest of the protein is dispensable for such binding. In other words, the DBD2 and/or DBD3 of HMG I(Y) specifically mediate the interaction to Crx. It appears that the affinity of DBD2 to Crx is the highest, followed by DBD3 and then DBD1 (comparing I15, I13, and I4). Altogether, these results in conjunction with the previous functional data suggest that HMG I(Y) proteins may participate in enhanceosome formation on the rhodopsin promoter by interacting specifically, via the DNA-binding domain, with Crx on the BAT-1 site.

DISCUSSION

Data coming from a variety of laboratories now indicate that the nonhistone chromosomal proteins HMG I and Y play critical roles in gene activation during embryogenesis and cell activation (for review, see Bustin and Reeves, 1996; Bustin, 1999). The proteins appear to contribute to gene activation by promoting enhanceosome formation via protein–protein and protein–DNA interactions. There is now clear evidence showing that HMG I(Y) proteins physically interact with both the involving transcription factor families and their *cis* elements, leading to facilitation of transcrip-

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tion factor binding to their *cis* elements and stabilization of such a higher-order complex (Yie et al., 1997, 1999).

To date, HMG I(Y) proteins have been implicated in gene activation during embryogenesis, cell activation, and tumorigenesis, because these are the situations in which HMG I(Y) proteins are found in abundance. Indeed, practically no HMG I-C protein and no or barely detectable levels of HMG I and Y proteins are found in normal, terminally differentiated cells (Chiappetta et al., 1996). Here we show that very high levels of HMG I(Y) proteins are expressed in normal, terminally differentiated photoreceptor cells. We have set forth the hypothesis that this expression may be related to the unusually high metabolic rate and protein synthesis activity of photoreceptor cells (Leon et al., 1990; Berman, 1991). Specifically, several genes involved in phototransduction undergo cyclical expression patterns that are under diurnal control. Such a demand for dramatic changes in gene expression could be met by a mechanism involving transcription. To test our hypothesis that HMG I(Y) expression in photoreceptor cells is linked to the induction of photoreceptor-specific genes, we identified both a cell system and a target gene to assess this possibility. In addition, we reasoned that once a cell system and target gene were defined, that experiments could be performed to begin to understand the molecular basis of HMG I(Y) contribution to photoreceptor gene expression.

We first determined that the commonly used retinoblastoma cells for photoreceptor research resemble photoreceptors with respect to HMG I(Y) expression. We also have obtained evidence that HMG I(Y) proteins are indeed critical for the expression of the best characterized inducible retinal-specific gene, the rhodopsin gene. In this promoter, HMG I(Y) proteins interact with a discrete site. This finding is quite different from what has been found in other HMG I(Y)-dependent genes such as interferon-β, HLA-DRA, and E-selectin (Du et al., 1993; Lewis et al., 1994; Whitley et al., 1994; Abdulkadir and Ono, 1995; Abdulkadir et al., 1995, 1998; Falvo et al., 1995; Thanos and Maniatis, 1995). In these promoters, multiple HMG I(Y) binding sites are spread throughout the proximal promoter mediating both DNA–protein and protein–protein interactions in the enhanceosome. In addition, the site where HMG I protein footprints overlaps with BAT-1, which is also the binding site for the paired-like homeodomain protein Crx. This result was particularly interesting from two standpoints. First, the overlapping nature of the HMG I(Y) and Crx binding sites immediately suggested the possibility that HMG I(Y) and Crx might bind cooperatively to the BAT-1 element. Indeed, we and others have previously shown that HMG I(Y) proteins facilitate interaction of POU-homeodomain proteins with their binding sites (Abdulkadir et al., 1995, 1998; Leger et al., 1995). Second, the recent demonstration that mutations in Crx result in some cases of cone–rod dystrophy (CRD), Leber congenital amaurosis (LCA), and retinitis pigmentosa (RP) strongly suggests that Crx interaction with the rhodopsin promoter is critical for photoreceptor development and function (Freund et al., 1997, 1998; Swain et al., 1997; Jacobson et al., 1998; Morrow et al., 1998; Sohocki et al., 1998; Swaroop et al., 1999). The fact that we observed significant downregulation of the rhodopsin promoter by antisense HMG I(Y) RNA is consistent with a critical role for both HMG I(Y) proteins and Crx in rhodopsin gene expression.

Our studies indicate that binding of HMG I(Y) proteins is crucial for proximal rhodopsin promoter activity, specifically during Crx transactivation. Finally, we demonstrated the direct physical interactions between HMG I(Y) and Crx and mapped to the DBD2 and DBD3 of HMG I(Y) responsible for such interaction. Because Crx structure and function are critical for vision (mutations in Crx cause several forms of CRD, LCA, and RP), our data also bring up the possibility that mutations in HMG I(Y) proteins may also impact photoreceptor function.

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