The retinoic acid signaling pathway is controlled essentially through two types of nuclear receptors, RARs and RXRs. Ligand dependent activation or repression of retinoid-regulated genes is dependent on the binding of retinoic acid receptor (RAR)/9-cis-retinoic acid receptor (RXR) heterodimers to retinoic acid response element (RARE). Although unliganded RXR/RAR heterodimers bind constitutively to DNA in vitro, a clear in vivo ligand-dependent occupancy of the RARE present in the RAR/2 gene promoter has been reported (Dey, A., Minucci, S., and Ozato, K. (1994) Mol. Cell. Biol. 14, 8191–8201). Nucleosomes are viewed as general repressors of the transcriptional machinery, in part by preventing the access of transcription factors to DNA. The ability of hRXRa/hRARα heterodimers to bind to a nucleosomal template in vitro has therefore been examined. The assembly of a fragment from the RAR/2 gene promoter, which contains a canonical DR5 RARE, into a nucleosome core prevented hRXRα/hRARα binding to this DNA, in conditions where a strong interaction is observed with a linear DNA template. However, histone tails removal by limited proteolysis and histone hyperacetylation yielded nucleosomal RAREs able to bind to hRXRα/hRARα heterodimers. These data establish therefore the role of histones NH2 termini as a major impediment to retinoid receptors access to DNA, and identify histone hyperacetylation as a potential physiological regulator of retinoid-induced transcription.

Core histones H2A, H2B, H3, and H4 are the main protein components of chromatin around which DNA is wrapped in 146-bp1 segments, forming stable nucleosomal structures. Nucleosome spacing and histones post-translational modifications, and most notably acetylation, varies greatly from one chromosomal domain to another (with hyperacetylated histones being preferentially associated to transcriptionally active chromatin) and have strong effects on gene activity (reviewed in Refs. 2 and 3). Beside these long range effects, chromatin assembly on regulatory regions of eukaryotic promoters affects directly the transcriptional activity of genes. Due to the organization of these DNA sequences into precisely positioned arrays of nucleosomes, transcription factors access to their cognate response elements is in most cases restricted and nucleosome assembly is therefore viewed as a general cellular strategy to repress transcription. In vitro assembly of nucleosomes on short DNA fragments documented this type of effect for many DNA-binding proteins, including Gal4 (4), SP1 (5), nuclear factor 1 (6), heat shock factor (7), and TATA-binding protein (8), and genetic experiments in yeast using the PHO5 transcription unit have established a link between transcriptional activation and chromatin structure disruption (reviewed in Ref. 9). However, chromatin organization is also, in some instances, a mean to favor transcriptional activity of genes, as described for the estrogen-regulated vitellogenin B1 gene (10).

Therefore, transcription regulation must be viewed as a complex set of interactions involving specific transacting factors, general transcription factors, and coactivators recruitment onto a nucleoprotein complex. The productive interaction of DNA-binding proteins with their cognate response elements is thus conditioned by two parameters: (i) structural features of the nucleosome and (ii) dynamic processes leading to the alteration of chromatin structure by macromolecular complexes such as SWI/SNF (4, 11), which is associated to RNA polymerase II under low stringency conditions (12), nucleosome remodeling factor (13), or nucleoplasmin and NAP-1 (14). Structural features of chromatin also implies a competition between chromatin constituents and transcriptional coactivators (some of which bearing strong structural similarities with histones H3 and H4 (15)), which will eventually determine the overall transcriptional activity of genes (reviewed in Ref. 16).

In an effort to better understand the role of chromatin structure in nuclear receptors binding to their response elements, we have used purified components to investigate the impact of nucleosome assembly on a promoter containing a prototypical retinoic acid response element (RARE). Retinoic acid receptors (RARs and RXRs) heterodimers bind, in vitro, to RAREs with high affinity, irrespective of the presence of ligand. On the contrary, Ozato and co-workers (1) established, by in vivo footprinting experiments, that agonist treatment of target cells is an absolute prerequisite for heterodimers binding to the RARE of the RARβ gene, as it is to observe biological effects of retinoids in vivo. This agonist-dependent occupancy of hormone response element was also observed with the glucocorticoid receptor (17). The transcriptional and DNA binding activities of these nuclear receptors are therefore controlled in vivo at multiple levels, which include post-translational modifications (see

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‡ Present address: Laboratory of Molecular Growth Regulation, National Institutes of Child Health and Human Development, NIH, Bldg. 6, Rm. 2A11, Bethesda, MD 20892-2753.

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1 The abbreviations used are: bp, base pair(s); RARE, retinoic acid response element; RAR, retinoic acid receptor; RXR, 9-cis-retinoic acid receptor; DR5, direct repeat with a 5-bp spacer; T3, thyroid hormone response element; CBP, cAMP response element-binding protein; HAT, histone acetyltransferase; PAGE, polyacrylamide gel electrophoresis; ExoIII, exonuclease III.
for examples, Ref. 18 and 19) and by epigenetic mechanisms (reviewed in Refs. 20 and 21).

The transcripational activation observed in the presence of RAR and RXR is triggered by binding of heterodimers to RAREs that consist, in most cases, of a direct repeat (DR) of the sequence PuGGTCA. A direct repeat of the hexanucleotide PuG(T/G)TCA spaced by five nucleotides favors the binding of RXX/RAR heterodimers, whereas a spacing of four, three, or one base converts the direct repeat into a thyroid hormone, vitamin D, and 9-cis-RA or PPAR response element, respectively (Ref. 22, and reviewed in Ref. 23). RXR/RAR heterodimers display the highest affinity for half-sites spaced by 5 bp (DR5) and a lower affinity for half-sites having a 2-bp spacing. DR1 RAREs accommodate heterodimer binding in an opposite polarity (i.e., RXR being the 3′-bound receptor) (reviewed in Ref. 24). Nucleosome assembly on DNA sequences containing such direct repeats should therefore introduce two new types of constraints upon RXX/RAR heterodimer binding to DNA, as a consequence of the helical nature of the DNA. The first type of constraint is defined by the translational phasing of the nucleosome, which describes 5′ and 3′ boundaries of the octamer core on the linear DNA sequence and thus identify the dyad axis of the nucleosomal core particle. Transcription factors access to their cognate DNA-binding site is facilitated when protein-DNA interactions take place close to nucleosome boundaries, reflecting a looser interaction of DNA with histones, as demonstrated for the glucocorticoid receptor (25). The second type of constraint is the rotational phasing within the nucleosome core, which reflects the orientation of any segment of DNA relative to the core histone surface (26).

In this work, we have first examined the positioning of histone octamers in vitro on a retinoid-regulated promoter, the P2 promoter of the human retinoic acid receptor β (RAR-β2) that contains a DR5 response element (β-RARE (27)). We find that nucleosomes assembled spontaneously on DNA fragments from the RARβ gene P2 promoter at a position placing the β-RARE at the dyad axis of the core particle. Binding of purified hRARα/hRXRα heterodimers was prevented by the wrapping of the RARE around the native octamer. On the contrary, heterodimer binding could be observed when histone tails were removed by limited proteolysis and when histones were hyperacetylated, suggesting a critical role of this post-translational modification in the regulation of the DNA binding activity of retinoids receptors in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials**

Antiproteases, trypsin, and trypsin inhibitor were purchased from Sigma. *Taq* DNA polymerase was from Life Technologies, Inc. (Rockville, MD); isopropylthio-β-galactosidase, ampicillin, and kanamycin were from Appligene/Oncom (Strasbourg, France). Restriction enzymes and agarose were from Promega (Madison, WI), oligonucleotides were purchased from Eurogentec (Le Sart-Tilman, Belgium). Acrylamide and bisacrylamide mixture (Protogel) were from National Diagnostics (Atlanta, GA).

**Bacterial and Eukaryotic Cell Lines**

The JM109 bacterial strain was used for all subcloning procedures, whereas the M15 strain (Qiagen) was used for the overexpression of both His6-hRARα and His6-Flag-hRXRα (18). HeLa cells were used as a source for core histones and grown in Dulbecco’s minimal essential medium containing 10% fetal calf serum, penicillin, and streptomycin (100 units/ml) to 80–90% confluency prior to harvesting and histone octamer extraction.

**Core Histone Purification**

Core histones were prepared essentially as described in Côté et al. (32). The entire procedure was carried out at 4 °C. HeLa cells from 20 T25 flask were collected in 1 × phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.4) and washed twice with this buffer. 20 pellet volumes of buffer CLB (20 mM Tris-HCl, pH 8.0, 3 mM MgCl2, 0.25 mM sucrose, 0.5 mM phenylmethylsulfonyl fluoride) with 0.5% Nonidet P-40 were added and cells broken by 10–15 strokes in a Dounce homogenizer. Lysate was centrifuged for 20 min at 6,000 × g, and the nuclear pellet washed twice with the following buffer: 50 mM of buffer TNE-ep (10 mM Tris-HCl, pH 8.0, 0.4 mM NaCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 5 μl leupeptin) was added for 12 mg of DNA and the mixture was stirred gently for 15 min. Nuclei were pelleted and washed once with buffer TNE-ep, then resuspended in buffer PBso (50 mM NaPO4, pH 6.8, 0.6 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 5 μl leupeptin). While stirring, 20 μg of bovine serum albumin (Bio-Gel Bio-Rad) was added in the running buffer for gel-filtration using standard procedures (31). Histones were fractionated on 17% polyacrylamide, 0.9 μ acetic acid, 6.25 μ urea as described by Panyim and Chalkley (29) on 20-cm gels. Gels were run at 20 mA for 15 h and stained with Cooamassie Blue. In this system, the expected order of migration is H1-H3-H2A, H2B-H4 (from top to bottom).

**Retinoic Acid Receptors Purification**

Full-length hRARα and hRXRα were purified by metal chelate affinity chromatography to homogeneity as described previously (30). hRARα was expressed as an NH2-terminal fusion protein with a His6 tag, whereas hRXRα was fused to the His6 tag upstream of the Flag epitope (IBI-Kodak, Rochester, NY). hRXRα was eluted from the affinity matrix by enterokinase cleavage, thereby removing the His6 tag from the protein. Both receptors were expressed in the M15 *Escherichia coli* strain. Monoclonal antibodies directed against MRGS-His6 and Flag epitopes were purchased from Qiagen and IBI-Kodak, respectively.

**Plasmids and DNA Probes**

The bacterial expression vectors pQE9-hRARα and pQE9-His6-F-hRXRα have been described previously (18). Sequences containing the wild type β-RARE response element were isolated from pPro-RARβ (27) by restriction enzyme digestion. Milligram amounts of plasmid DNA were cut and selectively dephosphorylated at one end by calf intestine alkaline phosphatase (Promega), and the resulting insert was purified by agarose gel electrophoresis and electroelution. After phenol/chloroform extraction, DNA fragments were ethanol precipitated and quanified. 10 pmol were labeled with T4 polynucleotide kinase and purified by gel-filtration using standard procedures (31).

**Reconstitution of Nucleosomes**

Nucleosomes were reconstituted essentially as described in Ref. 32, except that the dialysis step was replaced by a serial dilution in buffer NRB (25 mM Tris-HCl, pH 7.4, 1.2 mM MgCl2, 5 mM β-mercaptoethanol, 5% glycerol) from 2 × NaCl to 1.5, 1.2, 0.8, 0.6, and 0.5 mM NaCl to reach a final volume of 200 μl. Typically, 50–100 ng of end-labeled probe (1–5 × 106 cpm, 300 fmol) were mixed with varying core histones amounts, so as to obtain a histone:DNA mass ratio ranging from 0.5 to 3, 0.5 μg of salmon sperm DNA, and 0.5 μg of bovine serum albumin. Naked DNA controls were obtained by performing similar dilutions in the absence of histones, which were added after the last dilution step. Aliquots of each reconstituted sample were analyzed on a 4.5% polyacrylamide gel in 0.5 × TBE (1 × TBE is 90 mM Tris base, 90 mM boric acid, 2 mM EDTA) and samples containing more than 90% of reconstituted octamer-DNA complexes were used in further experiments. The final concentration of nucleosome core particles was around 5–10 fmol/μl.

**DNase I and Exonuclease III Protection Assays**

Reconstitutes or naked DNAs were treated with either DNase I or exonuclease III prior to DNA extraction and resolving of DNA fragments on 6% urea denaturing polyacrylamide gels. 15 μl of reconstitution or control mixtures (about 30,000–40,000 cpm) were brought to 5 mM MgCl2 and CaCl2 and 1 unit of DNase I (Worthington, Freehold, NJ) was added. Digestions were for 0 to 8 min at room temperature (–22 °C), and stopped by addition of 100 μl of stop buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS). 10 μg of proteinase K was
Fig. 1. Core histones purification and nucleosome assembly on the RAR-β2 promoter. A, purification of core histones from HeLa cells. Protein content of the hydroxyapatite column eluate (50 µL) was subjected to 15% SDS-PAGE fractionation and proteins were visualized by Coomassie Blue staining. MW, molecular masses (indicated on the left); C, native histones. B, oligomeric states of purified core histones. 50 µg of purified histones were incubated in the presence of 1 mg/ml dimethyl suberimidate at pH 7.4 in 2 mM NaCl for 30 min at 20 °C. The reaction was quenched by 1 M Tris-HCl, pH 7.4, and samples resolved as in A. Positions of histone octamers, tetramers, and dimers are indicated on the right. MW, molecular masses (indicated on the left); C, native histones; X, cross-linked histones. C, reconstitution of nucleosome cores with fragments of the RAR-β2 promoter. Top panel, structure of the RAR-β2 promoter and localization of DNA fragments used in reconstitution experiments: DNAs containing sequences originating from the RAR-β2 promoter were excised using the indicated restriction enzymes. Functional cis-acting sequences are indicated as follows, from left to right: white arrow, imperfect DR5 RARE; black arrow, β-RARE DR5; empty box, TATA box; +1, transcription initiation site. Lower panel, electrophoretic mobility shift assays of reconstitutes generated with labeled DNA probes and increasing amounts of purified histones were performed as described under “Experimental Procedures.” F, free DNA; numbers indicate the histone dilution factor used. In these conditions, a dilution factor of 1:1 would correspond to a histone:DNA mass ratio of 0.8. Mononucleosome formation results in the complex labeled “Nuc.”.

Other Techniques

Western Blotting Procedure—Proteins were resolved on a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. Immunodetection of Hist-hRARα and/or FhRXRα complexes was performed as described previously using the IBI BioMax system (19).

Protein Assay—The protein content of receptor preparations was assayed by the Bradford assay (34) using bovine serum albumin as a standard.

Sequencing Reactions—Sequencing reactions were run using 32P-labeled primers and dideoxynucleotides mixes according to the manufacturer’s instructions (Amersham/U. S. Biochemical Corp.). Sequencing reactions were run using the native pPro-RAREβ plasmid as a template, yielding DNA ladders extending beyond the 5′ and 3′ ends of the DNA fragment used in reconstitution experiments.

RESULTS

Nucleosome Assembly on DNA Fragments from the Promoter of the Human RARβ Gene—In this study, we used purified core histones from HeLa cells (Fig. 1, A and B) to reconstitute nucleosome core particles following dilution from high salt. As shown by SDS-PAGE fractionation of core histones, histone H1 concentration was less than 3% of total histones (Fig. 1A). Histone oligomers were further characterized by cross-linking to characterize the octameric structure of purified histones. The main cross-linked band appeared to be an octamer, indicating that our starting material for reconstitution experiments is indeed a stable core nucleosome. DNA fragments used in nucleosome reconstitution experiments contain a RARE organized as a direct repeat of two hexanucleotides separated by 5 bp (DR5). An imperfect DR5 RARE (DR5) is also found 14 bp upstream of this RARE, whereas the TATA box is located 6 bp downstream of the DR5 sequence. Additional cis-acting elements are also present in this sequence (Fig. 4). Labeled DNA fragments of varying length (326, 240, and 182 bp, Fig. 1C) containing these functional cis-acting elements were used to investigate whether they can assemble into a nucleosome core in the presence of histone octamers. Similar amounts of each DNA fragment were thus used as a template for nucleosome assembly with increasing amount of histones. As expected, DNA fragments above 200 bp generated complexes displaying discrete electrophoretic mobilities, indicative of the association of several histone octamers on these DNAs, or of distinct octamer positioning (Fig. 1C). More interestingly, the 182-bp frag-
Translational and Rotational Phasing of the Reconstituted Mononucleosome—To examine whether this product has features of native nucleosomes, we first optimized reconstitution procedures. The result of a typical experiment yielding more than 90% of the naked probe reconstituted in nucleosome core particles is shown. In this case, a histone:DNA mass ratio, which was found to be optimal in the 0.8 to 3.0 range in our conditions (Fig. 2A). Boundaries of the core particle were determined by the exonuclease III protection assay (Fig. 2B). This enzyme digests from the 3′ end to the 5′ end of DNA and its progression along the sequence is strongly, but not totally, inhibited by proteins bound to DNA. Thus translational positioning of the reconstituted nucleosome can be analyzed by the ExoIII assay (Fig. 2B). The protection pattern of the labeled top strand indicated a major histone-induced ExoIII stop at position +32 (see Fig. 4 for sequence numbering), with weaker protections being observed at positions +42, +52, +62. On the contrary, no stop to ExoIII progression could be detected for the 5′-labeled lower strand, suggesting that histone octamers position preferentially at the 5′ end of this particular DNA fragment. Thus these data define a −150-bp segment on which nucleosome cores adopt a preferential, but not unique, positioning. The nucleosomal structure of this complex was further characterized by DNase I experiments (Fig. 3). DNase I cleavage sites (which correspond to a maximal accessibility of the DNA minor groove) on reconstitutes were clearly different from these of naked DNA. A periodic pattern extended from −101 to +2 on the upper strand, but the −61 to −20 segment was consistently found to be less accessible to DNase I cleavage for reasons that are not clear to us. Thus DNase I-generated fragments were amplified by a linear polymerase chain reaction (Fig. 3B) to fully characterize the rotational positioning of the double helix over the DR5 RARE and TATA box sequences. Again, a highly 10–11 bp periodic cleavage pattern was observed, typical for a DNA fragment organized around the surface of a histone octamer. This repetitive pattern was also clearly and reproducibly detected on the bottom strand in reconstituted DNA fragments on a stretch extending from −87 to +24 (Fig. 3C). Thus the DNase I pattern, together with 5′ and 3′ boundaries defined by the ExoIII protection assay, strongly suggest that a histone octamer is positioned from −112 to +32, with the DR5 RARE lying across the dyad axis of the nucleosome and demonstrate that this precise DNA segment has an intrinsic structure directing a precise translational and rotational positioning of the histone octamer. These features are summarized in Fig. 4.

DNase minor groove cleavage sites indicate that the third and fourth bases of each half-site of the DR5 RARE are highly accessible in the reconstituted mononucleosome. Since, according to molecular modeling (35), DNA-binding domains of RXR and RAR make extensive contacts with the major groove of the first three or four bases of each half-sites, we conclude that major contact points are facing toward the octamer, and therefore are in an orientation unlikely to be compatible with heterodimer binding to the RARE.

Importance of Histones NH₂ Termini in RAR/RXR Heterodimer Binding to Nucleosome Core Particles—To test this hypothesis, full-length, E. coli-expressed hRXRα and hRARα were purified to homogeneity using a Ni2⁺-nitrilotriacetic acid affinity matrix (Fig. 5A) and their ability to bind to a 20-mer oligonucleotidic probe containing the DR5 RARE was assessed by the electrophoretic mobility shift assay (Fig. 5B). Cooperative binding of purified RXR/RAR heterodimers to this response element was consistently observed in these conditions, indicating that a large fraction of the purified polypeptides is functional and binding to the core RARE with an affinity in the nanomolar range. Binding of purified heterodimers to the DR5 RARE present in the 182-bp DNA fragment used in mononucleosome reconstitution experiments occurred with a similar efficiency (Fig. 6, lanes 1–7). The nature of each complex was characterized by supershift experiments using monoclonal antibodies directed against theNH₂-terminal tag of each receptor. The electrophoretic mobility of DNA-RAR/RXR complexes was clearly decreased in the presence of each immunoglobulin (lanes 8 and 9), demonstrating that RAR/RXR heterodimers are indeed formed in these conditions. This DNA template was therefore assembled into a nucleosome core and tested similarly for its ability to bind RAR/RXR heterodimers. As predicted from the rotational orientation of both half-sites, this template did not accommodate RXR/RXR dimers, even in conditions where more than 90% of the naked probe was bound.
RXR/RAR Heterodimers Binding to Nucleosomal DNA

Fig. 3. Rotational phasing of the 182-bp RAR-β2 promoter fragment. A, DNase I footprint of the upper strand of the RAR-β2 promoter. Free DNA (F) or reconstituted monosomes (R) were incubated for 0, 1, 2, or 5 min or 0, 2, 5, or 10 min, respectively, with 1 unit of DNase I at room temperature. DNAs were extracted and analyzed as described in the legend to Fig. 2. Black dots indicate sites of enhanced DNase I sensitivity of nucleosomal DNA compared with free DNA. Positions of preferential DNase I cleavage were determined at the base pair level using dideoxynucleotides sequencing reactions (lanes G, A, T, and C). Numbers indicate the sequence position of cleavage sites along the promoter sequence. B, polymerase chain reaction amplification of the +2/–112 DNA segment. DNase I-digested DNA was amplified with a 19-mer oligonucleotide complementary to the upper strand. Fragment sizing was carried out using the Kodak 1D Image Analysis Software and results are indicated on the right. Corresponding cleavage sites by DNase I on the upper strand are indicated on the left. C, DNase I footprint of the lower strand of the RAR-β2 promoter. Free DNA (F) or reconstituted monosomes (R) were cleaved and analyzed as in A. Open circles show less intense, but consistently observed, cleavage sites. Positions of maximal minor groove accessibility (DNase I hypersensitive sites) were deduced from sequencing tracks and are indicated on the left. Experimental data are summarized in Fig. 4.

Fig. 4. Sequence of the wild-type RAR-β2 promoter fragment used in reconstitution experiments. Assignment of nucleosome boundaries and rotational phasing. A summary of the exonuclease III and DNase I protection patterns shown in Figs. 2 and 3 on the wild type RAR-β2 promoter are shown. Sequence has been truncated at 5′ and 3′ boundaries of the nucleosome (+112 to –34). Dots indicate the nucleosome-specific DNase I-hypersensitive sites detected on the coding strand, whereas triangles depict DNase I-hypersensitive sites detected on the noncoding strand. Positions of the imperfect DR5 element, as well as that of the canonical DR5 RARE and of the TATA box are indicated between the two strands. Additional cis-acting elements such as a cAMP-response element (–99 to –92), a AP-1 response element (–84 to –78), and an Inr element (–8 to +5) have also been characterized in this promoter.

(compare lane 5 to lane 14). 10-Fold higher receptor concentrations were also used and failed to evidence an interaction of these nuclear receptors with nucleosomal templates (data not shown). We conclude from these experiments that the organization of retinoic response elements around an histone octamer prevents, in our system, the binding of hRXRα/hRARα heterodimers to a prototypic response element.

Amino-terminal regions of histones are hyperacetylated in transcriptionally active chromatin. A direct link between these two processes has now been established (reviewed in Ref. 36–38). This post-transcriptional modification can also be mimicked in vitro by removal of histone tails by trypsin (see Ref. 39 and references therein). We wished to determine whether histone tail removal or histone hyperacetylation could influence binding of RXR/RAR to nucleosomal templates. Therefore, we treated purified core particles with trypsin or extracted core histones from HeLa cells treated with 10 mM sodium butyrate, a potent inhibitor of histone deacetylase activities. Electrophoretic analysis of both histones preparations evidenced the expected structural alterations: limited proteolysis of core histones yielded polypeptides migrating more rapidly than native histones in SDS and acid-urea polyacrylamide gels (Fig. 6A). Sodium butyrate treatment generated histone species that migrated more slowly in SDS-polyacrylamide gels (Fig. 6B), and
Acid-urea gels characterized at least 7 species with distinct electrophoretic mobilities that were different from those appearing with native histones. This pattern is compatible with the hyperacetylation of histones H3 and H4 which are highly acetylated and usually form triple bands in such a system. These two types of preparation were thus used in reconstitution experiments as described above. Both sources of core histones yielded nucleosome particles with the -112/+70 RARβ P2 promoter fragment, with a slightly increased electrophoretic mobility observed with trypsinized histones. Reconstitutes were then tested for their ability to bind RXR/RAR heterodimers. We observed that histone tails removal by limited trypsin treatment allowed the binding of receptor heterodimers to the mononucleosomal DNA. The specificity of heterodimer binding to the nucleosomal DR5 RARE was assessed by competition with a 100-fold excess of unlabeled DR5 oligonucleotide, which was able to displace the complex. The addition of the anti-RAR antibody disrupted this complex, suggesting that the conformation of the ternary complex RAR/RXR-nucleosomal RARE is distinct from that of the binary complex RAR/RXR-RARE (compare lane 8 to lane 26). On the contrary, the anti-RXR IgG increased the molecular mass of the ternary complex as observed for the naked DNA fragment, showing that the NH₂-terminal end of RXR is still fully accessible in this complex as observed for the naked DNA fragment, showing that this DNA fragment has structural properties facilitating DNA bending and therefore curving around the histone octamer. DNase I protection experiments carried out on the wild type DNA reconstituted around native core histones evidenced a typical cleavage pattern alternating every 10 ± 2 base pairs, and allowed the determination of the rotational setting of the wild type DNA. Minor grooves of both half-sites were found to be oriented facing away from the octamer, forming a closed recognition interface for RXR/RAR heterodimers. Indeed, Rastinejad and colleagues (35) reported that RXR/thyroid hormone receptor (T3R) DBDs dimers, and by extension RXR/RAR dimers, engage the major grooves of the successive half-sites. In contrast, the minor groove of the TATA box was found to be sensitive to DNase I digestion, evidencing a proper exposure of the TATA-binding protein-binding site (40). However, this translational and rotational setting of the TATA box has proven to provide a poor substrate for TATA-binding protein binding within the TATA-binding-protein-binding site (40). In keeping with these observations, we found that the nucleosome-assembled DR5 response element did not allow cooperative binding of RXR/RAR dimers, in opposition to naked DNA. Glucocorticoid receptor is known to bind to its cognate response elements as a homodimer structurally close to RXR/T3R and RXR/RAR DBDs dimers. Wrange and colleagues (25) reported that a glucocorticoid response element having a analogous rotational setting (with minor grooves pointing away from the histone octamer) is unable to bind glucocorticoid receptor dimers. By analogy, we would predict that modifying the rotational setting of the DR5 RARE would render the nucleosomal template has been reported to occur both in vivo and in vitro (42). RXR/T3R binding was, however, in this system, thyroid-hormone independent; ligand binding led to an alteration of the chromatin structure and coincidental transactivation of the promoter. Alteration of the rotational setting of the wild type T3RE was found be detrimental for the binding of RXR/T3R heterodimers to nucleosomal DNA (43). Ozato and colleagues (1, 44) reported a stringent ligand-dependent occupancy of the RARE of the RAR-β2 promoter in vivo, as well as other cis-acting elements of the promoter. This
occupancy occurred coincidentally to promoter activation, without inducing major remodeling of the nucleosomal structure of this promoter. This observation is in striking contrast with in vitro assays showing that RXR/RAR dimers bind constitutively to RAREs (i.e. in a ligand-independent manner) (for a review, see Ref. 21, and references therein). We postulate that, based on our observations, these discrepancies could be explained by the specific architecture of the RAR-β2 promoter leading to a closed conformation of the DR5 element in a nucleosomal context, although its in vivo organization remains to be precisely characterized.

If RXR/RAR heterodimers are excluded from nucleosomal DNAs in our system, then the nucleoprotein structure of this locus has to be altered in some way to allow for RXR/RAR recruitment. Histone hyperacetylation is the most common post-translational modification targeted to the NH₂ termini of these proteins. The reversible charge neutralization of highly conserved lysine residues by acetyl groups reduces the capacity of histones tails to stabilize the path of DNA along the octamer core. Consequently, allosteric changes are thought to occur in the nucleoprotein complex and renders the nucleosomal DNA more accessible to transcription factors (39), although a role in the disruption of nucleosome-nucleosome interactions can be predicted from recent crystallographic data (45). Histone tails clipping in vitro by limited trypsin treatment mimicks the effects of hyperacetylation and facilitate GAL4, TATA-binding protein, and TFIIIA binding to “chromatinized” DNA (39, 41, 46). To ascertain whether this post-translational modification could influence RXR/RAR heterodimers binding to nucleosomal DNA, we used both trypsinized histones and histones extracted from sodium butyrate-treated cells in reconstitution experiments (Fig. 6). Our results demonstrate that both sources of core histones yielded nucleosome core particles able to bind receptor heterodimers, identifying histone hyperacetylation as a major control event in the regulation of retinoid receptors access to DNA. We note that the yeast coactivator Gcn5p has been identified as the catalytic subunit of a histone acetyltransferase (HAT) (47). More relevant to the studied phenomenon is the characterization of a mammalian Gcn5 homologue, p/CAF (48). This protein has been shown to interact with CBP/p300, a co-activator interacting with T3R, RAR, and RXR (49) and to stimulate the transcriptional activity of progesterone and estrogen receptors (50). CBP/p300 is also a coactivator for a number of transcription factors such as cAMP response element-binding protein, AP-1, MyoD, and c-myc (51–53). Very importantly, CBP/p300 has been shown to possess intrinsic HAT activity (54, 55), suggesting that RXR/RAR binding to RAREs could direct HAT(s) to retinoic acid-regulated promoters. Although transcriptional synergy with CBP/p300 is generally characterized using transiently transfected templates for which the importance of the chromatin structure is questionable (56), this is not exclusive of its involvement in the coordinate recruitment of transcriptional regulators altering chromatin structure. Two other nuclear receptor coactivators, SRC-1 and ACTR, have also been shown to possess histone acetylase activity (57, 58). Thus several HAT activities may be tethered to hormonally-regulated promoters by liganded receptors and act synergistically. A possible cooperativity between two dis-
tinct chromatin-modifying complexes is underlined by the physical interaction between the ADA5pGen5 complex and the SWI-SNF complex (36), which potentiates glucocorticoid receptor-mediated transcription in yeast (59) and for which mammalian counterparts have been identified (60). Consistent with these observations, the histone deacetylase HDAC1 was found to be associated with SMRT, a nuclear receptor corepressor binding to unliganded RARs (61), and inhibition of cellular deacetylase activities by trichostatin A led to potentiation of retinoic acid-induced transcription and cellular differentiation (62, 63). All the studies above therefore establish a direct link between nuclear receptor-mediated transcriptional activation and histone acetylation.

Several predictions can be made from these and our data, assuming that retinoid-controlled promoters are organized in nucleosomal arrays: (i) histone hyperacetylation will be of more or less importance to retinoic acid-induced transcriptional activation, depending on the rotational and translation positioning of the response element. As a consequence of the observed in vitro positioning of the RAR-β2 promoter, we would predict a strong dependence of its transcriptional activity on HAT activities. (ii) CBP and/or associated HAT (pCAF, Gen5p homologues) and other factors yet to be identified are likely candidates for chromatin-dependent RXR/RAR coactivators. Transcription factors like E1A (59), or agents (anti-AP1 retinoids (64)) known to modulate CBP-retinoid receptors interaction may have a direct influence on HAT activities targeted to retinoid-controlled promoters, and thus on RA-mediated transcriptional activity. Further experiments in progress in our laboratory comparing the in vitro and in vivo organization of RA-controlled promoters will provide new insights into the importance of chromatin remodeling in retinoid receptor-mediated transcriptional activation, in conjunction with the use of powerful molecular tools such as specific ligands and mutated receptors.

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