Introns of the chicken ovalbumin gene promote nucleosome alignment *in vitro*

James D. Lauderdale⁺ and Arnold Stein^{*}

Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA

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ABSTRACT

A defined *in vitro* chromatin assembly system was used to examine the nucleosome alignment induced by histone H5 throughout a 12 kilobase pair chicken genomic DNA fragment containing the ovalbumin gene. In contrast with total fragmented chicken DNA and several anonymous cloned fragments, much of the gene permitted histone H5 to space nucleosomes at physiological intervals in an extended array. Nucleosomes at the 3'-end of the gene and on approximately 4 kilobase pairs of 5'-flanking ovalbumin sequence did not become aligned to appreciable extents. Analysis of cloned 2–3 kilobase pair subfragments suggested that a strong nucleosome alignment signal, specifying a 196 ± 5 base pair repeat exists in intron E. A second discrete region of the gene, which mapped approximately to intron A, exhibited nucleosome alignment with a spacing periodicity of about 200 base pairs. The ovalbumin cDNA did not permit nucleosome alignment. These findings suggest that some of the introns contain signals that direct nucleosome alignment over the ovalbumin gene in a way conducive to its regulation.

INTRODUCTION

Eukaryotic DNA requires packaging into chromatin, and it is reasonable to suspect that there might be information present in the base sequence that influences this process. One type of signal might affect precise nucleosome positioning through variations in the 'bendability' of different base sequences (1). Sequence analysis has revealed subtle dinucleotide periodicities in eukaryotic DNA supporting this idea (2–4). The precise positioning of nucleosomes with respect to factor-binding sites in transcriptional promoters could be functionally important (5). There is also evidence for subtle periodic structural variations in DNA on a larger scale that might influence the formation of whole arrays of nucleosomes (6–8). Signals such as these that could influence the packaging of regions of chromatin might also be functionally important. For example, all regions of DNA might not become packaged into a highly ordered inert chromatin structure with equal efficiency. Thus, DNA control regions, which are often found to have a less ordered or distinctive

structure (9, 10), might be less efficiently packaged, allowing regulatory proteins to compete with histones more effectively (11–12). It has not been possible to determine whether less ordered chromatin regions exist to facilitate factor binding, or if such regions arise merely as a consequence of factor binding.

Recently, we found that chromatin assembly *in vitro* (the generation of a highly ordered, physiologically spaced nucleosome array) in a defined, histone H1-dependent system, required a particular DNA region (approximately 1000 bp) fortuitously present in the plasmid pBR327. Once nucleated, a highly ordered chromatin structure could spread for several kb onto adjacent plasmid or eukaryotic sequences that by themselves did not undergo nucleosome alignment. Moreover, this fortuitous plasmid signal, which we have referred to as a chromatin organizing region (COR), may somehow encode the 210 bp nucleosome spacing periodicity observed for the chromatin assembled. The nucleosome repeat length was not affected by the core histone to DNA ratio, by adding excess linker histone, or by changing the linker histone type from H5 to H1 (13). The COR base sequence, unfortunately, has no distinguishing features, and we have not as yet been able to determine how it nucleates the nucleosome alignment reaction.

Because of these findings, it was of interest to examine eukaryotic DNA and attempt to find signals with properties similar to those of the COR. Such signals might not be very common because total mixed sequence DNA fragments prepared from either chicken erythrocyte or hen oviduct do not assemble into chromatin well in our *in vitro* system. Poor nucleosome alignment was also found upon examination of several purified cloned chicken DNA fragments, from randomly chosen clones (unpublished observations). We have found signals that promote histone H5-dependent nucleosome alignment in cloned chicken ovalbumin DNA, and here present an analysis of 12 kb of continuous DNA containing the chicken ovalbumin gene.

MATERIALS AND METHODS

Nucleosome formation and alignment

Histones, both core and H5, were prepared from chicken erythrocyte nuclei as previously described (13). Nucleosome formation on supercoiled plasmid DNA and nucleosome alignment using histone H5 in a buffer containing 0.15 M NaCl

* To whom correspondence should be addressed

⁺Present address: Department of Biology, University of Michigan, Ann Arbor, MI 48109, USA

and sodium polyglutamate, as well as micrococcal nuclease (MNase) digestion and electrophoresis were as previously described (13). Typically, 54 μ g of core histones were added to 60 μ g of DNA in an NaCl solution such that the final DNA concentration was 200 μ g/ml and the final NaCl concentration was 1.0 M before dialysis. Thus, a core histone to DNA ratio of 0.9 (wt/wt) was used for nucleosome reconstitutions unless otherwise noted. Only supercoiled plasmid DNA was used in this study. The nucleosome alignment reactions contained 0.5 μ g histone H5/ μ g DNA for all samples. Control reactions were incubated under identical conditions except no histone H5 was present. The criteria for determining nucleosome alignment and measurement of the nucleosome spacing periodicity have been described previously (14, 15).

Plasmid constructions

Plasmids pOv230, containing a 1959 bp insert of ovalbumin cDNA (16), and pOV12, with a 12 kb DNA fragment containing the entire ovalbumin gene, (17) were obtained from Dr B.O'Malley's laboratory. The insertion of a 2.3 kb fragment of anonymous chicken erythrocyte DNA fragment (designated A13) into the BamHI site of pBR327 was described previously (14). DNA fragments from pOV12 were subcloned using standard procedures (18). pOV12 was digested with the appropriate restriction enzyme(s) following the manufactures recommended conditions, and the DNA fragments were separated on 5% polyacrylamide gels in TBE buffer (89 mM-Tris-borate (pH 8.0), 2 mM Na₂EDTA). Fragments of interest were then cut from the gel and electroeluted (ISCO apparatus). The isolated fragments were purified by extraction four times with phenol/chloroform/isoamyl alcohol (24:24:1), ethanol precipitated, and dissolved in 0.2 mM Na₂EDTA. The DNA fragments were, where appropriate, incubated with the Klenow fragment of *E. coli* DNA polymerase to make the ends of the molecules blunt, purified by phenol/chloroform/isoamyl alcohol (24:24:1) extraction, precipitated with ethanol, and dissolved in 0.2 mM Na₂EDTA. DNA fragments subcloned into the EcoRI site of pBR322 were mixed with purified EcoRI digested pBR322 which had been blunt ended using the Klenow fragment of *E. coli* DNA polymerase, and ligated. DNA fragments subcloned into the EcoRV site of pBR327 were mixed with purified EcoRV digested pBR327 and ligated together. After transformation of either HB101 or DH5 α , colonies were screened for the appropriate insert by either colony hybridization (18) using the appropriate ³²P-labeled purified DNA fragment as a probe, or selection for antibiotic sensitivity (18). All constructions were verified by restriction mapping. Subfragment a was the 3523 bp XbaI-XbaI fragment from the 5' end of the ovalbumin sequence. Subfragment b was the 2697 bp SspI-SspI ovalbumin fragment. Subfragment c was the 1901 bp PvuII-PvuII ovalbumin fragment. Subfragment d was the 1901 bp NdeI-NdeI ovalbumin fragment. Subfragment e was the 2063 bp XbaI-EcoRI ovalbumin fragment. A 102 $\pm$ 7 bp deletion was made in the pBR322 construct containing subfragment c by using Exonuclease III and nuclease S1. The Exonuclease III reaction was carried out at 23°C to slow the rate of nucleotide removal to approximately 100 nucleotides/min. The deletion was made bidirectionally from the ClaI site in pBR322.

After transformation of DH5 α , colonies were screened for various extents of deletion. Constructs were sized by analysis of the DNA fragments generated after digestion with EcoRI + BamHI, RsaI, or HaeIII. The DNA fragments were sized on 1.5% or 2.0% agarose gels run in TBE buffer. The gels were

calibrated using 123 bp ladders (BRL). DNA fragments from EcoRI + BamHI, RsaI, or HaeIII restriction digests of the undeleted construct were also used for calibration of the gels. All constructs were prepared for nucleosome reconstitution as described (13).

Southern blotting/hybridization

Hybridization of DNA was carried out as described (13) except that in all cases 100 ng of DNA was loaded per agarose gel lane. DNA restriction fragments from the chicken ovalbumin gene were used to probe pOV12, subfragments a, b, c, and d in the EcoRI site of pBR322, and pOv230. These were: the 970 bp EcoRI-BglI fragment (probe '1'), the 183 bp PstI-SspI fragment (probe '1a'), the 210 bp BglI-EcoRI fragment (probe '2'), the 235 bp HinfI fragment (probe '3'), the 344 bp DdeI fragment (probe '4'), and the 202 bp SnaBI-XbaI fragment (probe 'exon 5'). DNA restriction fragments from pBR327 were used to probe constructs containing subfragment c in the EcoRV site of pBR327 or a 2.3 kb fragment of anonymous chicken erythrocyte DNA (A13) in the BamHI site of pBR327. These were the 191 bp BamHI-SphI fragment (375-566 on the pBR327 map) and the 218 bp TaqI-TaqI fragment (1268-1486 on the pBR327 map).

RESULTS

Histone H5 induces nucleosome alignment over most of the ovalbumin gene

A map of the 12 kb chicken genomic DNA fragment containing the ovalbumin gene is shown in Figure 1. This gene has 8 exons, the last of which contains 647 untranslated nucleotides, and 7 introns of varying lengths (19-21). Core histones were first added to this 12 kb fragment contained in pBR322 forming irregularly spaced and close packed nucleosomes. Then the sample was incubated with histone H5 in the presence of sodium polyglutamate in a buffer containing 0.15 M NaCl. These conditions have resulted in extensive nucleosome alignment (extending over 3 kb), with a 210 bp spacing periodicity, in constructions that contained the COR from pBR327 (13). In pBR322, used here, the COR is interrupted and functions poorly (13). Moreover, the 12 kb insert is sufficiently large so that its possible influence by plasmid DNA should be minimal. The local nucleosome alignment throughout the chicken DNA insert was assessed by Southern blotting, using probes separated by 2-3 kb (indicated in Figure 1). Figure 2 shows the results of this analysis.

Figure 1. Map of the 12 kb chicken genomic DNA fragment in pOV12, subfragments, and probes. The solid line denotes the chicken DNA insert containing the ovalbumin gene; the dashed lines denote plasmid DNA. The HindIII (Hd) sites mark the end of the chicken sequence. EcoRI (RI) and BamHI (Bm) sites present in pBR322 are indicated. The dark boxes indicate exons 1-7; letters A-G identify the corresponding introns. Positions of hybridization probes 1, 1a, 2, 3, 4 and subfragments a-d are indicated.

Proceeding across the insert from left to right, probes 1 and 1a indicated that nucleosomes aligned poorly over the first 3–4 kb, corresponding to 5'-flanking DNA. In contrast, probe 2 from intron A detected a regular nucleosome array extending for at least 2.5 kb, with a spacing periodicity of 202 ± 5 bp. Similarly, probe 3 from intron E/exon 5 detected a strong 196 ± 5 bp periodicity, closely resembling that found for native chicken oviduct chromatin (22). Neither the naked DNA nor chromatin containing only core histones generated MNase digests resembling native chromatin. Probe 4 from the 5'-end of exon 7 detected little nucleosome alignment. These data indicate that histone H5 induced nucleosome alignment over most of the ovalbumin gene, but not on the flanking DNA or exon 7. In this

type of experiment, the DNA in the vicinity of the hybridization probe is assessed. The more extensive digest surveyed chromatin structure within about 1000 bp of each probe, while the less extensive digest surveyed within about 2–3 kb of each probe. The precise boundaries of the ordered region cannot be deduced from this data.

Analysis of nucleosome alignment on 2–3 kb cloned subfragments

Additional information was obtained by examining subcloned DNA fragments obtained from the original 12 kb insert (indicated in Figure 1). Figure 3 shows that subfragments b and c, 2.7 and 1.9 kb, respectively, each aligned nucleosomes, whereas,

Figure 2. Nucleosome alignment induced by histone H5 in pOV12 chromatin. Southern blots of MNase digests obtained using probes 1 (A), 1a (B), 2 (C), 3 (D) and 4 (E) are shown. Lanes 1 and 2 show 1 and 2 min digests, respectively, using 5 units MNase/ μ g DNA under nucleosome-aligning conditions. Lanes R show 30 sec digests of samples that lacked histone H5. Lanes D show naked DNA digested under the same conditions, but with 3 units MNase/ μ g DNA for 80 sec. Lanes M contained 32 P-labeled HaeIII + AccI Φ X174 RF DNA size markers; lengths (bp) are indicated. In (C) and (D) selected nucleosome oligomer bands, which are multiples of a unit repeat, are indicated.

Figure 3. Nucleosome alignment induced by histone H5 on various 2–3 kb subfragments. Lanes 1 and 2 show 2 and 3 min digests, respectively, using 4.2 units MNase/ μ g DNA for samples assembled into chromatin under nucleosome-aligning conditions. Lanes R show 1 min digests of samples that lacked histone H5, and lanes D show 50 sec digests of naked DNA under the same conditions. Lanes M contained markers, as described in Figure 2. The label m identifies the monomer of the nucleosome ladder. Southern blots were hybridized to the central regions of cloned subfragments b (A), c (B), a (C), and d (D), using probes 2, 3, 1a, and 4, respectively.

subfragments a and d, 3.5 and 1.9 kb, respectively, did not. A possible explanation for the failure of the terminal regions of the 12 kb insert (Figure 2) to align nucleosomes is the close proximity of these regions to plasmid DNA. However, the fact that alignment was obtained with subfragments b and c shows that the presence of plasmid DNA close to the chicken DNA insert does not in general inhibit alignment. Also, the failure of subfragments a and d to align nucleosomes provides a control showing that the alignment detected is dependent upon the insert being tested, and is not due to alignment merely on flanking plasmid DNA. Additionally, the alignment observed around intron E in the large construct (Figure 2) did not require the presence of intron A and *vice versa*. To determine whether alignment signals are discrete elements or are distributed uniformly throughout the gene, a subfragment (e) that partially overlapped b and c was analysed. This subfragment, which contained exons 1–4, the smaller introns B, C, D, and a 343 bp portion of intron E (see Figure 1), did not align nucleosomes to a significant extent (not shown). Thus we have found evidence for the existence of two discrete nucleosome alignment signals in the ovalbumin gene, one within or near intron A and another within or near intron E.

Alignment on a subfragment is not dependent upon a particular phase relationship with plasmid sequences

Although the results described above rule out a general inhibitory effect of proximal plasmid sequences, the possibility existed that sufficient signals were still present in pBR322 DNA to interfere with signals that might be present in all eukaryotic inserts. Thus nucleosome alignment on eukaryotic inserts might only occur when these two signals were in phase, and the 'phase angle' would have been arbitrarily chosen by the location of the restriction site used to prepare the subfragment. Nucleosome

Figure 4. Assessment of whether the relationship between sequences in the insert and in the plasmid influences nucleosome alignment within the insert. Samples were assembled into chromatin and digested for 2 min with 4.2 units MNase/ μ g DNA. Lane 1 shows the ladder obtained from a sample containing an approximately 102 bp deletion of DNA sequences at the subfragment c-plasmid junction; lane 2 shows the ladder from the undeleted construct. Southern blots were hybridized to probe 3 from the center of subfragment c in each case. Nucleosome oligomer bands are identified at the left of the photograph. Lane M contained 32 P-labeled size markers, as described in Figure 2.

spacing and ordering could be detected on pBR322 DNA near the BamHI site (see Figure 1) in some constructs (data not included).

To test this possibility, we chose subfragment c, because it exhibited very good alignment. If this alignment were dependent

Figure 5. Influence of subfragment c on nucleosome alignment on adjacent plasmid DNA in pBR327. Samples were assembled into chromatin and digested with 5 units MNase/ μ g DNA. (A) Southern blot of 1 min digest; the region between the insert and the COR was probed. For lane 1 the insert was ovalbumin subfragment c. For lane 2 the insert was an anonymous chicken DNA sequence of approximately the same length; multiples of the 210 bp unit repeat are identified. (B) Ethidium bromide stained gel for the sample containing subfragment c. Lanes 1–3 show 1, 2, and 4 min digests, respectively. Lane CE shows native chicken erythrocyte chromatin; multiples of the 207 bp unit repeat are identified. Lane M contained a 123 bp ladder; selected lengths are indicated. (C) Southern blot of the same samples shown in (A), but probed using a sequence from the COR. Multiples of the 210 bp repeat are identified.

upon fortuitous constructive interference between the signal in the fragment and a signal in the plasmid, it should be abolished by altering the phase relationship by one half of the unit repeat, approximately 100 bp. In this case, the non-aligning subfragments might be expected to align nucleosomes upon making the same alteration. To alter the subfragment c-plasmid junction, approximately 29 bp were removed from the subfragment and the cloning site was moved 73 bp closer to the region of the plasmid that aligned nucleosomes. In this way a phase alteration of 102 bp was made without deleting appreciable chicken DNA. Figure 4 shows that nucleosome alignment on the chicken DNA was not affected by this alteration. Therefore, the presence or absence of alignment on a eukaryotic DNA fragment should reflect its intrinsic properties.

More detailed analysis of the subfragment containing intron E

It was clear that some regions of ovalbumin DNA can align nucleosomes, but it was not clear whether these regions had properties similar to the pBR3237 COR. Specifically, it was not clear whether a single ovalbumin gene region could influence nucleosome alignment on adjacent chromatin. Unfortunately, with our current knowledge, it was not straightforward to examine these properties directly because all common (high copy) cloning vectors contain the pMB1 replication origin, which makes up a portion of the COR. The presence of a portion of the COR might interfere with the analysis. Moreover, any DNA fragments which might be inserted as a buffer between the pMB1 origin and the test fragment would need thorough characterization to insure that they lacked signals but were responsive to alignment signals present in adjacent DNA. The results were informative, however, when subfragment c was examined in pBR327, with the COR intact.

Subfragment c contained all of intron E (958 bp) localized toward one end of the fragment. This subfragment was inserted into pBR327 such that the intron E-end was about 800 bp away from the end of the COR from which nucleosome alignment appears to propagate. The nucleosome alignment induced by histone H5 in the region of the plasmid between the COR and the ovalbumin insert was examined using a plasmid DNA probe. As a control, an anonymous chicken genomic fragment (clone A13), inserted into the same region of pBR327, was examined. Figure 5A shows MNase ladders for the two samples, detected using the same plasmid hybridization probe, for samples run on the same gel in adjacent lanes. It can be seen that the A13 anonymous fragment insertion permitted an extended ladder to be detected (lane 2). The nucleosome spacing periodicity was 210 ± 5 bp, the value characteristic of the COR (13), suggesting that nucleosome alignment spread from the COR, over the intervening plasmid DNA, and onto the insert. It was previously shown that the order detected on this construct required the presence of the intact COR (14). For the ovalbumin insert, an extended periodic ladder was also detected (lane 1), but, upon comparison with the adjacent ladder, it is clear that the nucleosome repeat length was shorter than 210 bp. The repeat detected on plasmid DNA for the ovalbumin construct was 196 bp, as observed on ovalbumin DNA in the 12 kb insert in pBR322. This result suggests that order propagated from the ovalbumin fragment over the plasmid DNA, overpowering the influence of the COR and conferring the 196 bp ovalbumin repeat on the same sequence that previously was packaged with a 210 bp repeat in constructs lacking ovalbumin DNA. Moreover, the 196 bp ladder could be detected on total (5.2 kb, plasmid plus insert) DNA by ethidium bromide staining (Figure 5B), and over most plasmid regions, using several probes (data not included).

Figure 6. Influence of subfragment c orientation on nucleosome alignment on adjacent plasmid DNA in pBR327. Assembled chromatin was digested with 5 units MNase/ μ g DNA for 2 min (lanes 1, 2) or 3 min (lanes 3, 4). The Southern blot was hybridized to a 191 bp plasmid DNA sequence from approximately the center of the 787 bp region between the insert and the end of the COR. In one orientation (lanes 1 and 3) intron E was closer to the region of the plasmid probed than in the other orientation (lanes 2 and 4). Multiples of the 196 bp unit repeat are indicated at the left of the photograph. Marker sizes are indicated at the right.

Figure 7. Nucleosome alignment on ovalbumin cDNA. Samples were assembled into chromatin and digested with 4.2 units MNase/ μ g DNA for 2 min. The Southern blot was hybridized to a 202 bp sequence taken from exon 5, approximately in the center of the cDNA (lane 2) and the subfragment c (lane 1) inserts. Multiples of the 196 bp unit repeat generated from subfragment c are indicated at the left of the photograph.

However, when the plasmid COR was probed, for the same chromatin assembly reactions, a 210 bp ladder was observed for the A13 construct, but poorer ordering was detected for the ovalbumin construct (Figure 5C). This result is consistent with the ovalbumin signal clashing with the plasmid signal in this region. These data suggest that this ovalbumin subfragment contains a strong signal, capable of conferring a 196 bp spacing

on adjacent chromatin, and thus has properties similar to those of the COR.

Examination of the nucleosome alignment properties of subfragment c inserted into the same cloning site of pBR327 as before, but in the other orientation, further suggested that the signal in this subfragment might be in intron E. In this experiment the same plasmid junction hybridization probe was used as above. Here, however, the other end of the subfragment, containing exons 5 and 6 (separated by the 323 bp intron F) was adjacent to the plasmid junction previously probed. Figure 6 shows that the subfragment orientation had a significant effect on the MNase ladder detected in the vicinity of this junction. In the previous orientation (lanes 1 and 3), the 196 bp ladder was detected, as before, but in this orientation (lanes 2 and 4), a longer 210 bp repeat was detected, similar to that observed for the anonymous chicken DNA insert (Figure 5). Comparison of lanes 1 and 2 shows the nucleosome oligomer bands going out of phase with each other upon proceeding up the two ladders, just as expected for this repeat length difference. The longer (210 bp) repeat for the present orientation can be explained if the plasmid region being probed was in this case more strongly influenced by the COR because it was now closer to it than to the signal in intron E, which specified the shorter repeat.

Ovalbumin cDNA does not align nucleosomes

The data obtained so far was consistent with the idea that nucleosome alignment signals might reside in some of the larger introns of the gene. In order to test this hypothesis further we examined alignment on the 1.9 kb cDNA, contained in pBR322, using a fragment from exon 5 as a hybridization probe. Figure 7 shows that alignment on the cDNA was poor, compared to that detected on subfragment c, using the same probe. Although it cannot be concluded from this experiment that coding DNA is devoid of nucleosome alignment information, this result is consistent with the idea that the presence of introns facilitates nucleosome alignment over the gene.

DISCUSSION

We have presented evidence that all regions of a eukaryotic genome (chicken) are not equivalent with regard to DNA packaging into chromatin. Whereas total fragmented chicken DNA and several randomly chosen cloned fragments failed to exhibit histone H5-induced nucleosome alignment *in vitro* (unpublished observations), most of the ovalbumin gene aligned nucleosomes well. Discrete alignment signals appear to be present in the gene in two of the large introns. This conclusion is based upon the failure of the cDNA to align nucleosomes (Figure 7), and the dominant effect of intron E on nucleosome alignment on adjacent plasmid DNA, in contrast with the passive properties of exons 5 and 6 (Figure 6). Alignment also occurred on the subfragment containing intron A, but not on the fragment containing small introns B, C and D. It could not be determined from these data if an additional signal exists in intron G.

Interestingly, the 5' and 3' flanking regions of this gene did not undergo nucleosome ordering. One possibility is that these flanking DNA regions are resistant or refractory to nucleosome ordering. Alternatively, these regions may not be within range of the alignment signals. Exon 7 is about 3 kb downstream of the midpoint of intron E, which appears to contain a signal that influences nucleosome alignment bidirectionally. The disordered

5'-flanking region is closer to intron A, but this signal might be weak or directed downstream. The pBR327 COR appears to be a unidirectional signal (13). It could not be determined from these data if the intron A signal is unidirectional as well.

The nucleosome alignment signals present in the ovalbumin gene functionally resemble the COR, the fortuitous alignment signal found in plasmid pBR327 (13). Intron E was investigated most thoroughly here: neither of these signals has as yet been precisely localized. Both signals appear to nucleate nucleosome alignment in a histone H5 (or H1)-dependent fashion. Once nucleated, the alignment can spread over adjacent regions of chromatin, not capable of generating ordered physiologically spaced nucleosome arrays on their own. For example, the experiments described in Figure 5 show that much of the plasmid DNA was packaged into chromatin with a 196 ± 5 bp nucleosome spacing periodicity when the plasmid contained intron E. This is apparent even by total DNA staining (Figure 5B), where it can be seen that the spacing periodicity is shorter than that of native chicken erythrocyte chromatin (207 ± 5 bp) (22). In the absence of the intron E fragment, the degree of nucleosome alignment or the repeat length was clearly different from that observed here, in a variety of different plasmid constructions (13, 14).

One difference between the intron E signal and the COR was very apparent. These two signals nucleate the assembly of chromatin having different repeat lengths. This can be seen, perhaps, most clearly in Figure 5A where the ladders are side-by-side. Under the influence of only the COR, a 210 bp repeat was generated, whereas under the (stronger) influence of intron E, a 196 bp repeat was generated. In this experiment all reaction components were identical, except for the DNA insert in the plasmid, and it was plasmid DNA that was probed. This result provides strong support for our previous suggestion that the nucleosome repeat length of the chromatin assembled can be specified by the nature of the chromatin organizing signal (13). As we found before, the repeat length was unaffected by changing the nucleosome density on the DNA, or by adding excess histone H5. Specification of the nucleosome repeat by the use of particular chromatin organizing signals in DNA is a novel hypothesis for how repeat length differences in different species and tissues (23) and in different chromosomal loci of the same cell (24–27) might arise.

The *in vivo* relevance of signals in DNA that affect nucleosome alignment *in vitro* remains to be established. There is evidence that introns in some genes play a role in facilitating transcription by a mechanism not consistent with the presence of enhancer elements within the intron. It has been found that a large number of cDNA-based constructs are expressed poorly in transgenic mice (28, 29), and that the presence of homologous introns significantly increased transcriptional efficiency (29, 30). Although the mechanism whereby homologous introns facilitate transcription is not known, our finding that some introns contain signals that promote nucleosome alignment supports the hypothesis that sequences in introns may alter nucleosome–DNA interactions and consequently determine whether a functional transcription complex is assembled at the promoter (30). A curious observation was that this effect was not found when the same constructs were transfected into cultured cells (29). In transformed cells (used for the transfection experiments), the bulk nucleosome spacing periodicity is generally found to be about 10 bp shorter than in animal tissues (23) and what we have observed *in vitro*, suggesting that an overall rearrangement of

chromatin structure has occurred. Possibly, the base sequence-dependent mechanism specifying nucleosome alignment *in vitro* is operative in animal tissues, but altered or overridden in transformed cells in culture. This would provide an explanation for the differences observed in the effect of introns on gene transcription in animal tissues and in cultured cells.

The pattern of nucleosome alignment on the ovalbumin gene and flanking DNA that we observed *in vitro* may closely resemble that of native chromatin in chicken oviduct, the tissue in which this gene is expressed. First, the nucleosome spacing periodicity in hen oviduct is 196 ± 5 bp (22), virtually the same as what we have observed here. Second, in chicken oviduct, the 5' and 3' flanking DNA for several kb exhibit poor nucleosome alignment (31, 32), similar to that observed here, even though these regions are not transcribed. In hen oviduct, poor MNase ladders were also observed within the ovalbumin gene, but this is clearly a consequence of ongoing transcription in this region. In the immature chick, perturbation of nucleosome alignment within the gene was directly related to the level of hormone-induced transcription, and a regular 196 bp repeat was observed in hormone-withdrawn chicks (33). It has been argued that the poor nucleosome alignment observed in the chromatin flanking the gene cannot be attributed to an intrinsic property of the DNA because typical ladders were detected in chicken erythrocytes (32). However, the uniform packaging of this whole DNA domain into a chromatin structure characteristic of the bulk chromatin in erythrocytes, with the unusually long 210 bp repeat, might be a consequence of an unusual chromatin packaging mechanism related to the 'heterochromatization of the nucleus' that occurs in avian erythrocytes (34), or the use of different DNA packaging signals which are dominant. MNase digests have apparently not been probed in flanking regions of the ovalbumin gene in tissues other than oviduct and erythrocyte.

In another hormone-inducible gene of the chick, the lysozyme gene, the pattern of DNase I hypersensitive sites (DH sites) has been examined in detail in a variety of tissues (see reference 10 for a review). Since DH sites generally represent discontinuities in the nucleosome array (10), it can be inferred that in most cases nucleosome alignment detected by MNase in the vicinity of a DH site would be poor (irregular). It is interesting that four DH sites (two 5' and two 3') flanking the lysozyme gene have been detected in liver, kidney, and brain (but not erythrocyte), tissues where the gene is not expressed (35, 36). These data therefore suggest that the nucleosome arrays flanking the chick lysozyme gene are irregular in most tissues, consistent with what we observed for chromatin assembled *in vitro* on chicken ovalbumin DNA. Although it is premature to make generalizations, it is plausible that the nucleosome alignment observed on ovalbumin DNA might reflect an arrangement common to some hormone-inducible genes.

Irrespective of whether nucleosome alignment in the chromatin flanking inducible genes is in general irregular, our data suggests that signals present in some of the larger introns of some genes strongly modulate DNA packaging. In the case of the chicken ovalbumin gene, signals in the DNA are such that the regions flanking the gene should be expected to be at least more resistant to packaging into a typical nucleosome array than the gene itself, and, presumably, to other regions of the genome. The flanking regions, which contain recognition sequences for protein factors, therefore, would then be able to compete much more effectively for these protein factors than readily packaged DNA regions.

It is very plausible that in some cases the presence of precise nucleosome positioning with respect to regulatory protein binding sites could be responsible for the irregular nucleosome arrangement. This packaging mechanism might be important for discouraging nonspecific or inappropriate factor binding, and in controlling the expression of some inducible genes.

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