In Vitro Assembly of the Characteristic Chromatin Organization at the Yeast PHO5 Promoter by a Replication-independent Extract System*

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An extensive set of analyses of the yeast PHO5 gene, mostly performed in vivo, has made this gene a model for the role of chromatin structure in gene regulation. In the repressed state, the PHO5 promoter shows a characteristic chromatin organization with four positioned nucleosomes and a short hypersensitive site. So far the basis for this nucleosome positioning has remained unresolved. We have therefore decided to complement the in vivo studies by an in vitro approach. As a first step, we have asked whether the characteristic PHO5 promoter chromatin structure depends on the cellular context including replication or higher order nuclear chromatin organization or whether it can be reconstituted in vitro in a cell-free system. To this end we have established an in vitro chromatin assembly system based on yeast extracts. It is capable of generating extensive regular nucleosomal arrays with physiological spacing. Assembly requires supplementation with exogenous histones and is dependent on energy leading to chromatin with dynamic properties due to ATP-dependent activities of the extract. Using the PHO5 promoter sequence as template in this replication independent system, we obtain a nucleosomal pattern over the PHO5 promoter region that is very similar to the in vivo pattern of the repressed state. This shows that the chromatin structure at the PHO5 promoter represents a self-organizing system in cell-free yeast extracts and provides a promising substrate for in vitro studies with a direct in vivo correlate.

Eukaryotic cells assemble their DNA with histone and non-histone proteins into a complex structure called chromatin. All nuclear, DNA-related processes therefore have to contend with chromatin as their primary substrate. Elucidating the structural and regulatory role of chromatin is now widely recognized as an important part of the research on transcription, replication, recombination, and repair (1, 2).

Our laboratory is interested in the role of chromatin in gene regulation. In this regard, the PHO system in yeast has become a model system and here we focus on the PHO5 gene. In the repressed state, the PHO5 promoter region is organized into four positioned nucleosomes with a short hypersensitive site between nucleosomes −2 and −3 (Fig. 2). These four nucleosomes become remodeled as a prerequisite for the induction of the PHO5 gene leading to an extended nuclease hypersensitive site (3). Many aspects of this process have been elucidated by a series of in vivo studies. The chromatin transition is strictly dependent on the transactivator protein Pho4 but independent of replication and PHO5 transcription (4). Recently, the histone acetyltransferase Gcn5 as well as the nucleosome remodeling complex SWI/SNF were shown to have a kinetic effect on the promoter opening process, and the former could be linked to a transient hyperacetylation of the promoter histones (5, 6). The fully induced promoter was shown to be devoid of histones (6, 7), which provides an answer to the long standing question of the molecular makeup of a hypersensitive site (8).

Despite the fruitful and ongoing in vivo approach, there remain issues, especially mechanistic questions, which will be difficult to answer solely by analyzing whole cells. Therefore we set out to extend our study of the PHO system by a complementary in vitro approach. At the beginning it was not for certain whether such an approach would be feasible. It is not predictable so far whether a certain chromatin domain with its typical nucleosome positioning represents an autonomous structure or whether its assembly depends on the nuclear context including higher order chromatin folding, nuclear matrix organization and replication. Especially the first two would make an in vitro reconstitution system rather unlikely to generate the proper chromatin structure.

In order to address this point, we turned to in vitro chromatin assembly systems based on cellular extracts. In contrast to uncatalyzed systems like salt gradient dialysis or histone chaperone-mediated assembly, the cellular extracts contain enzymatic activities e.g. ATP-dependent remodeling machines, which might be vital for generating in vivo like nucleosome positioning. Several systems are currently available based on extracts from Xenopus eggs, Drosophila embryos, or mammalian cells (9–13). For the study of the yeast PHO system a homologous chromatin assembly using yeast extracts seemed desirable. However, at the time when we launched into this project the only yeast extract system described (14, 15) did not reliably produce extensive, regular nucleosomal arrays, which is a hallmark of physiologically relevant chromatin.

We found that this limitation can be overcome by the supplementation of yeast extracts with exogenous histones. Excitingly, besides supporting the regular spacing of arrays such a yeast extract based in vitro chromatin assembly system is able to generate the proper positioning of nucleosomes. Using the DNA sequence of the PHO5 locus as a template we demonstrate that a nucleosome pattern over the PHO5 promoter, which is very similar to the in vivo pattern, can be generated by de novo assembly in vitro. This is a proof of principle that the

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**EXPERIMENTAL PROCEDURES**

**Yeast Whole Cell Extract Preparation**—Our procedure is based on the protocol of M. C. Schultz (14) with modifications by S. E. Kong and J. Q. Svejstrup. A 2-liter culture of strain YS22 (MATα, his3-11, his3-15, leu2-3, leu2-112, can1, ura3Δ5, pho4::URA3) was grown in YPD at an OD600 of 2–4 and harvested at 3000 g at room temperature. The pellet was resuspended with 0.5 ml of extraction buffer containing CompleteTM without EDTA and dialyzed against this buffer. The dialysis need not go to completion but diminished the performance of the extract. The extracts have a protein concentration of 30–60 mg/ml as determined by Bradford assay with bovine serum albumin as standard.

**Chromatin Assembly**—For each preparation of DNA, extract, and histones, the ratio of DNA:extract:histones had to be carefully optimized. A 150-μl chromatin assembly reaction contained for example 2 μg of DNA, 300 μg of extract protein, and 6 μg of Drosophila histone octamers. The DNA template was the circular, supercoiled, 10.01 kb plasmid pCB/LEU2 (derivative of pCB/ wt (16) with the TRP1 marker replaced by the LEU2 marker). Drosophila histone octamers were purified essentially as described (17) and kept in 1 M KCl and 50% glycerol at −20 °C. The standard assembly buffer consists of 20 mM Hepes/KOH pH 7.5, 80 mM KCl, 25 mM (NH4)2SO4, 1 mM EDTA, 390 mM (NH4)2SO4, 1 mM MgSO4, 20% glycerol, 1 mM EGTA, 5 mM dithiothreitol, and CompleteTM without EDTA. After slow thawing, the slurry was centrifuged for 2 ha t100,000 g at 1°C. At least two freeze-thaw cycles did not diminish the performance of the extract. The extract has a protein concentration of 30–60 mg/ml as determined by Bradford assay with bovine serum albumin as standard.

**FIG. 1.** Generation of extensive, regular nucleosomal arrays by in vitro chromatin assembly with a yeast whole cell extract. Aliquots of a complete assembly reaction (lanes 7–18 and 20–22) as well as assembly reactions without supplementary histones (lanes 1–3) or energy (lanes 4–6) were analyzed after the indicated incubation time by MNase digestion. Wedges at the top reflect increasing MNase digestion. Lanes 23–25 show MNase digestion of nuclei and lane 19 the 123-bp ladder (Invitrogen) as size marker. The same blot membrane was hybridized with similar results to a probe for the coding region (shown) or for the promoter region (not shown) of the PHO5 gene.

no extra histones no energy complete yeast extract in vitro assembly system yeast nuclei

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The contribution of the histones regarding KCl and glycerol was taken into account. Siliconized tubes were blocked with a solution of 2 mg/ml bovine serum albumin, 0.1% Nonidet-40 for all assembly reactions. The assembly reaction was incubated at 30 °C. Prior to any manipulation or analysis, the assembly reaction was always centrifuged for 3 min at room temperature and maximum speed in a table-top centrifuge.

Gel Filtration and Sucrose Gradient Fractionation of Chromatin—150 μl of chromatin were loaded onto a 600-μl sucrose gradient (10–50% sucrose in assembly buffer), centrifuged for 1 h at 27,000 × g and 25 °C and fractionated. Fractions were screened for chromatin by Southern blotting. In addition or alternatively, 150 μl of chromatin were spun through 1 ml of Sephacryl S300HR (Amersham Biosciences, equilibrated in assembly buffer plus 0.2 mg/ml bovine serum albumin) for 1 min at 1100 × g and room temperature yielding the chromatin in the flow-through.

**Chromatin Analysis**—Nuclei preparation and chromatin analysis by restriction enzymes, MNase or DNase I as well as indirect end-labeling, gel electrophoresis, and blotting procedures were as described (18, 19). Secondary cleavage for DNase I digests employed ApxI and for restriction enzyme digests HaeIII. Probe 1 corresponds to the Apal-BamHI fragment upstream of the PHO5 gene and probe β to the HpaI-ClaI fragment at the beginning of the LEU2 gene. The BamHI-BstEII fragment was used as PHOS5 promoter probe and a PCR product using the primers GCCAACACTTTGAGTCTTGGAAC and TGGAGCTTGAAGAACAAGTTGGAACC as PHOS coding region probe. Quantification of restriction enzyme accessibility assays was done using a PhosphorImager (Fuji FLA3000, AIDA™ software).

**Apyrase Treatment and ATP Measurement**—Chromatin was incubated with 0.001 to 0.02 units/μl apyrase (grade I, Sigma) and incubated for at least 15 min at 30 °C. This treatment routinely reduced the ATP concentration by a factor of 10−5 to 10−6. The ATP concentration was measured with the luciferin/luciferase assay (Enlight3M, Promega; Lumat LB 9501, Berchtold Technologies). The assay was not inhibited by addition of a mock assembly without ATP.

**RESULTS**

The Yeast Whole Cell Extract Assembly System Generates Extensive Nucleosomal Arrays—Our in vitro chromatin assembly system uses a yeast whole cell extract. Its preparation was based on a protocol by Schultz (14) with an additional ammonium sulfate precipitation step. It was originally reported by Schultz (14) that yeast whole cell extracts failed to reliably produce extensive nucleosomal arrays like those seen with Drosophila embryo or Xenopus egg extracts (9–11). We found that this was due to a lack of sufficient amounts of endogenous histones in the yeast extract. Without additional histones, limited micrococcal digestion of a yeast extract assembly reaction indeed showed a smear only (Fig. 1, lanes 1–3). In contrast, if
the assembly reaction was supplemented with purified histones the yeast extract system was very capable of generating extensive arrays (Fig. 1, lanes 20–22). This assembly required energy in addition to supplementary histones. A reaction without the ATP regenerating system yielded only few closely spaced nucleosomes (Fig. 1, lanes 4–6). The third critical factor for successful chromatin assembly was the incubation time. The kinetics of the assembly reaction were slow. The length of the arrays increased steadily for up to 6 h when finally the pattern looked very similar to those found with nuclei in vivo (Fig. 1, lanes 20–22 versus 23–25). Interestingly, while the length of the arrays increased with assembly time, the spacing of the nucleosomes did not change with time but remained constant from the beginning (Fig. 1, lanes 7–18 and 20–22). Under the conditions shown here, the nucleosomal repeat length was about 170 bp but was to some extent dependent on the salt concentration (data not shown). Apart from the assembly without additional histones we did not observe significant amounts of subnucleosomal DNA even at early time points. Similar results were obtained when a different region of the plasmid was probed (data not shown).

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**Fig. 2.** Schematic model of chromatin at the PHO5 promoter region in the repressed state. The positioned nucleosomes are represented by circles and numbered from -5 to +1 with respect to the transcription start site (broad black arrow). Open circles correspond to the nucleosomes, which become remodeled upon induction of the PHO5 gene. The two black dots show the position of the two Pho4 binding sites. T stands for TATA box and HS for the short hypersensitive site. The restriction sites used in this work are indicated.

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**Fig. 3.** The in vitro assembled chromatin is dynamic in an ATP-dependent way because of factors intrinsic to the yeast extract. Yeast extract assembled chromatin was digested with the restriction endonuclease ClaI after 6–7 h of assembly either without further additions (untreated chromatin) or after additional treatment with apyrase or after removal of unbound lower molecular weight components by sucrose gradient and/or gel filtration. In the latter case, part of the chromatin was sarkosyl treated (0.05%, 5 min at room temperature) prior to the separation step and 2.5 mM ATP and/or yeast extract (30–60 μg of extract protein) was added before ClaI digestion as indicated. Error bars indicate the S.D. of at least three independent experiments.
The nucleosome positioning generated by the yeast extract assembly closely resembles the in vivo pattern. A. Chromatin was analyzed by DNase I digestion and indirect end labeling with secondary ApaI cleavage and hybridization with probe 1. The DNase I patterns of four independent in vitro chromatin assembly reactions (lanes 8 and 9, 10–13, 14 and 15) after 6–7 h of incubation at 30 °C are compared with the pattern of yeast nuclei (lanes 6 and 7). Lanes 10–13 show the analysis of the same assembly reaction, which was either run over a sucrose gradient (lanes 10 and 11) or a gel filtration column (lanes 12 and 13). DNase I patterns of incomplete in vitro assembly reactions are shown in lanes 1–4:

- Without supplementary histones (lane 1),
- without energy (lane 2), and without histones and extract (free DNA, lane 3 and 4).

DNase I concentrations in units/ml were 0.007 (lane 3), 0.015 (lane 4), 0.23 (lane 13), 0.5 (lanes 6 and 12), 1 (lane 7), 2 (lane 10), 4 (lane 11), 7 (lanes 1, 2, 14), 15 (lane 9), and 30 (lanes 8 and 15). Lane 5 shows digests with either Dral, ClaI, or BamHI (top to bottom) as size markers for the PHO5 promoter. The arrowhead points to the short hypersensitive site. The labeled ovals on the side of the gel represent the positioned nucleosomes over the PHO5 promoter region with the same numbering as in Fig. 2 B, probing the chromatin structure at the PHO5 promoter by restriction enzyme accessibility. Chromatin assembled for 6–7 h was treated with apyrase and then digested with the indicated restriction enzymes. After secondary cleavage with HaeIII, the sites in the PHO5 promoter were monitored with probe 1 and the site in the LEU2 open reading frame (ORF) on the same plasmid with probe 2. Error bars represent the variation of two independent experiments.

The in vitro assembly of yeast PHO5 promoter chromatin suggested that the ClaI site was nucleosomal in the assembled chromatin. The separation of the chromatin from unbound lower molecular weight compounds by sucrose gradient fractionation and/or gel filtration removed the energy but did not remove the ATP-dependent factors as mere re-addition of ATP after the separation step restored ClaI accessibility (Fig. 3, column 4). The activity of the ATP-dependent factors responsible for the dynamic behavior could be removed after
assembly by treatment of the chromatin with the detergent sarkosyl (21) prior to the separation step. In this case, re-addition of ATP alone was not sufficient to increase the ClaI accessibility significantly unless yeast extract as a source of the ATP-dependent and sarkosyl-sensitive factors was added as well (Fig. 3, columns 6 and 7). The ClaI accessibility under static conditions of the sarkosyl-treated chromatin (Fig. 3, column 5), was significantly higher than without sarkosyl (Fig. 3, columns 2 and 3), indicating a damaging effect of sarkosyl on the chromatin structure.

**The Nucleosome Positioning over the PHO5 Promoter as Assembled in Vitro Closely Resembles the *in Vivo* Pattern.**—The assembly of DNA into extensive regularly spaced nucleosomal arrays as well as the ATP-dependent dynamics of the resulting chromatin were promising signs indicating that the yeast extract system might be able to generate a physiological chromatin structure *in vitro*. In our case we were particularly interested to see if the yeast extract system could generate *in vivo*-like nucleosome positioning over the PHO5 promoter. A direct comparison of the *in vitro* chromatin structure with the *in vitro* assembled chromatin as assayed by DNase I digestion and indirect end labeling revealed very close similarities (Fig. 4A; lanes 6 and 7 versus lanes 8–15). The short hypersensitive site between nucleosomes −3 and −2 is longer than a typical linker region and leads to a strong band in the DNA I pattern *in vivo*. It is a hallmark of the nucleosomal organization over the PHO5 promoter (HS, Fig. 2; arrowhead in Fig. 4A; Ref. 18). This band was strong and broad and at the proper location in the *in vitro* chromatin as well. The site of preferential cleavage by DNase I in free DNA, which notoriously leads to a band at the position of the Clal marker fragment (Fig. 4A, lanes 3 and 4, Ref. 18)2 was well protected after the *in vitro* assembly speaking for the stable positioning of a nucleosome at this location, i.e. the −2 nucleosome. The position of the band corresponding to the linker region between the nucleosomes −4 and −3 also matched exactly. Its intensity was usually very strong (Fig. 4A, lanes 8–15), a feature that is also sometimes observed *in vivo* albeit less often (Fig. 4A, lanes 6 and 7). The two bands framing the position of the −1 nucleosome were slightly shifted *downstream* in the *in vitro* chromatin as compared with the *in vivo* pattern. Differences in the DNase I pattern further downstream of the promoter, i.e. in the upper part of the lane, reflect differences in the DNA sequence. For example, the open reading frame of PHO5 is followed *in vivo* by a hypersensitive site and then the PHO3 gene (18) whereas the *in vitro* template harbors the CEN6 element here.

The proper positioning of the nucleosomes *in vitro* was again dependent on supplementary histones and energy (Fig. 4A, lanes 1 and 2) and was clearly distinct from the DNase I pattern of free DNA (Fig. 4A, lanes 3 and 4). The strikingly *in vivo*-like DNase I pattern was highly reproducible, examples from six independent assembly reactions with different extract preparations are shown in Figs. 4A, 5, and 6. We emphasize this point because achieving reproducibility was a major difficulty in setting up this assembly system. It demanded the appropriate buffer conditions and careful titration of template DNA, histones, and extract. Once assembled, the pattern persisted through sucrose gradient fractionation or gel filtration (Fig. 4A, lanes 10–13).

In addition to analysis by DNase I digestion and indirect end-labeling, the nucleosome structure over the PHO5 promoter was also probed by restriction enzyme accessibility under static conditions, *i.e.* after apyrase treatment (Fig. 4B). This technique has the advantage of covering the total population of DNA molecules whereas DNase I digestion with indirect end labeling may reflect a subpopulation due to the low degree of digestion intrinsic to the method. In *vivo*, the enzymes BamHI and BstEII cut in linker regions with accessibilities of about 50% whereas the site for ClaI lies within the −2 nucleosome and has a low accessibility of about 10–20% (Figs. 2 and 3).2 The up and down of the restriction enzyme accessibilities along the PHO5 promoter which arises from the positioned nucleosomal array was well recapitulated by the *in vitro* assembled chromatin. Regarding the absolute accessibility values, the value for the BstEII site appeared to be somewhat too low. This could be a consequence of the slightly shifted −1 nucleosome or reflect a heterogeneity of chromatin configurations or be a property of the BstEII enzyme. Different restriction enzymes tend to cope differently with chromatin as substrate, and even under dynamic conditions different restriction enzymes cleave to different extents (data not shown). This was also true for the *in vitro* assembled chromatin, although only when fully assembled. DNA assembled without exogenous histones was cut to 100% by all restriction enzymes tested (data not shown). This ruled out an inhibition of the enzymes by components of the extract or the buffer conditions of the assembly. In order to get a more meaningful perspective on the absolute accessibility values, we took advantage of a BstEII site in the open reading frame of the LEU2 gene on the same plasmid template DNA. The LEU2 BstEII site was 4-fold less accessible than the site in the PHO5 promoter (Fig. 4B). We took this lower value to represent an upper limit for a nucleosomal BstEII site. This in turn showed that the BstEII site in the PHO5 promoter region was relatively accessible and indeed represent a linker region.

**Nucleosome Positioning Slowly Matures into the Native Pattern over the PHO5 Promoter.**—The DNase I pattern in the region of the PHO5 promoter matures strikingly with time. We monitored the chromatin structure over the PHO5 promoter during assembly kinetics by DNase I digestion and indirect end labeling (Fig. 5). At early time points (Fig. 5; lanes 1–3), the region corresponding to the −2 and −3 nucleosomes exhibited multiple DNase I bands indicating multiple nucleosome positions. After 3.5 h and even more pronounced after 6.5 h the DNase I bands merged into few bands and formed the distinctive pattern of the PHO5 promoter (Fig. 5, lanes 6–8). We could literally watch how the −2 and −3 nucleosomes of the PHO5 promoter homed in on their proper positions during the *in vitro* assembly. Interestingly, the −1 or TATA-box nucleosome was inaccessible to DNase I right from the beginning even though this region is accessible in free DNA (Fig. 4A, lanes 2 and 4) speaking for a rapid positioning of this nucleosome. The pattern after about 6 h represented the equilibrium state as further prolongation of the assembly up to overnight did not lead to changes of the pattern, even if supplemented with additional creatine phosphate and creatine kinase (data not shown).

The pattern was also not influenced whether the DNase I digest was performed in the presence of ATP or after energy depletion by apyrase treatment (Fig. 6). This is due to the low degree of digestion, which is intrinsic to the DNase I indirect end-labeling method and ideally results in only one double strand cut in the region of interest. This limit digest will always score only the positions, which are occupied with high probability and the nucleosomes apparently become frozen in these positions after removal of energy by apyrase.

**DISCUSSION**

We have successfully established a cell-free, replication-independent *in vitro* chromatin assembly system based on yeast extracts that is able to position nucleosomes over the PHO5 promoter region very much as found *in vivo*. This shows that

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nuclear features like higher order chromatin folding (23), nuclear matrix or chromosome environment as well as the process of replication (24) do not play an essential role in setting up the nucleosome positioning at the PHO5 promoter. Instead, the PHO5 promoter DNA is able to act as the template for an ATP-dependent self-organization process if provided with a yeast extract and sufficient histones.

Our here established system provides a new tool for the study of nucleosome positioning and chromatin remodeling. It combines the advantage of a system thoroughly characterized in vitro with the possibility of asking mechanistic questions in vitro that cannot be addressed in vivo. The potential of such a system was recognized several years ago, but initial attempts using yeast extracts were unable to generate nucleosomal arrays as assayed by limited MNase digestion (14, 15). We found that this limitation was caused by the lack of sufficient amounts of endogenous histones in the yeast extracts. If supplemented with exogenous histones the extracts contained all activities needed to deposit histones and to align nucleosomes onto a DNA template in an ATP-dependent manner leading to extensive regular nucleosomal arrays. The need for supplementation with histones was described also for some Xenopus or Drosophila extract systems (9, 11). Very recently, Robinson and Schultz (25) also obtained nucleosomal arrays by adding exogenous histones and modifying the yeast extract preparation.

A hallmark of extract based in vitro chromatin assembly systems are physiologically spaced nucleosomes instead of the tightly packed core particles which are typically the result of salt dialysis methods. The same is true for a yeast extract system. Under the conditions shown here, we generate a repeat length that is even somewhat longer than found in vivo (170 versus 160 bp; (26)). However, the repeat length in extract based in vitro assembly systems is not a fixed parameter as it is influenced by the ionic conditions. Blank and Becker (27) could tune the repeat length between 165 and 191 bp by increasing the concentration of KCl from 6 to 90 mM in a Drosophila extract assembly system. In keeping with this, Robinson and Schultz (25) report a shorter spacing of 160 bp in their system, which has a lower ionic strength (10 mM Hepes, pH 7.5, 10 mM KOAc and 3 mM MgCl2; assuming ATP to sequester equimolar amounts of Mg2+) than our system (20 mM Hepes, pH 7.5, 80 mM KCl and 1.5 mM MgCl2).

Shimamura et al. (28) reported a chromatin assembly system using Xenopus egg extracts in which the nucleosomal repeat length decreased during the assembly kinetics, which was interpreted to be caused by more and more nucleosomes being loaded onto the DNA with increasing assembly time. In contrast to this and similar to observations with the Drosophila extract system (29), we found that the spacing in a given assembly reaction was constant right from the early time points on, and only the length of the arrays increased with prolonged assembly time. This could point to a highly cooperative assembly process, which starts in patches with the final nucleosome density and proceeds to align more nucleosomes along the DNA in the course of continuing assembly.

Beyond the generation of regular arrays, for us the most important question was whether the in vitro generated positions of the nucleosomes at the PHO5 promoter resembled those adopted in vivo. In the light of prior studies by other groups using cellular extracts for in vitro chromatin assembly this was not necessarily to be expected. It is common to use more or less positioning prone templates, e.g. containing strong nucleosome positioning sequences like the 5 S rDNA, or to rely on transcription factor binding to generate nucleosome positioning in extract based in vitro systems. Assembly with Xenopus egg extract for example positioned a nucleosome over the 5 S rDNA sequence (Ref. 28, although see Ref. 30). In contrast, the Drosophila embryo extract did not even recognize the 5 S rDNA positioning information (29). Instead, in the overwhelming majority of previous studies a specific DNA
binding factor was needed to generate a defined nucleosome structure with extract assembly systems. In an ATP-dependent manner DNase I hypersensitive sites and positioned nucleosomes were generated in response to the binding of a variety of factors like GAGA factor, TTF, Gal4-VP16, HNF3-related factor, or R3 lac repressor (31–35). Only a few cases showed nucleosome positioning in extract assemblies without obvious factor binding. The Drosophila hap26 gene was assembled with properly positioned hypersensitive sites but with a less strictly positioned nucleosome in between (36). The origin of this chromatin structure remained unclear, and the authors speculated about “the relative contributions of DNA sequence, secondary structure and putative, yet unrecognized DNA-binding proteins.” The in vitro-like nucleosome positioning over the MMTV promoter region was achieved in the Drosophila extract assembly system prior to addition of NF1 or other factors (37). Later reports on the other hand pointed to unorganized nucleosomes over the MMTV promoter in the uninduced state in transfected Xenopus oocytes which became positioned only after binding of the agonist-loaded glucocorticoid receptor (38).

The PHO5 promoter is not known to contain strong nucleosome positioning sequences as efforts to generate the right nucleosome positioning by salt gradient dialysis were unsuccessful. Furthermore, so far no protein factor has been recognized which would be responsible for the nucleosome positioning. Therefore, our choice of the PHO5 promoter as template for the in vitro chromatin assembly system poised the actual challenge of this project. It was not clear whether the assembly system together with the DNA sequence would contain sufficient positioning information or whether the nuclear context would be necessary. All the more exciting is our demonstration that a yeast whole cell extract is able to generate the characteristic nucleosome organization over the PHO5 promoter.

Although the in vitro generated nucleosomal pattern over the PHO5 promoter is very similar to the in vivo structure we note that there are subtle differences. The −1 nucleosome appeared slightly shifted in the downstream direction, and the linker between nucleosomes −3 and −4 was more pronounced in vitro than in vivo. However, as this emphasis on the region between the −3 and −4 nucleosome is sometimes found in vivo as well and as high resolution exonuclease III mapping of this linker with in vivo chromatin showed pronounced microheterogeneity in the positioning around this linker region, we take this feature as inherent in the system.

Robinson and Schultz (25) have compared recombinant yeast histones and histone octamers purified from Drosophila embryos in their yeast extract assembly system and report better results for the generation of nucleosomal arrays with Drosophila histones. So far we have used only Drosophila histones and the successful generation of the in vitro-like pattern shows that the source of histones cannot be crucial at this level.

In our view, the question remains open to what extent the DNA sequence and/or DNA binding factors or chromatin remodeling activities provide the determinants for nucleosome positioning. The extract may contain a positioning factor or the ATP-dependent remodeling machines may read out positioning information from the DNA sequence, which is not apparent in salt dialysis experiments. Further experiments are underway addressing these questions.

In contrast to our notion, Terrell et al. (39) report the generation of in vivo-like nucleosome positioning over the PHO5 promoter using an uncatalyzed in vitro chromatin assembly system based on the histone chaperone NAP1. They stress the role of DNA sequence as the determinant of nucleosome positioning. However, the positioned −2 nucleosome as inferred in this system did not affect the binding of Pho4 to its intranucleosomal binding site. This chromatin configuration is in contradiction to our in vivo data (40) and awaits further clarification.

The proper nucleosome positioning over the PHO5 promoter in our in vitro assembly system required careful titration of DNA, histones and extract in the appropriate buffer conditions. Even with the right conditions the assembly kinetics were slow. Apart from complicating the practical aspects of this system, this finding may have biological significance. The energy minima, which lead to specific nucleosome positions over the PHO5 promoter may be rather shallow with respect to competing positions. Even though the nucleosomes of the PHO5 promoter represent a classical example of reliably positioned nucleosomes in vivo, they may also represent a “loaded spring,” which opens up easily in response to a trigger, in this case to Pho4 binding. This concept would explain the old observation that PHO5 belongs to the most strongly induced genes in yeast (41) and would also fit with the recent finding that the induced promoter is not only devoid of positioned nucleosomes but also of histones altogether (6, 7).

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