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An upstream transcription factor, USF (MLTF), facilitates the formation of preinitiation complexes during *in vitro* chromatin assembly

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During *in vitro* chromatin assembly the formation of transcription complexes is in direct competition with the assembly of promoter sequences into nucleosomes. Under these conditions the fold stimulation of transcription by an upstream transcription factor (USF) was greater than that observed in the absence of nucleosome assembly. Function of USF during nucleosome assembly required the simultaneous presence of the TATA box binding protein TFIID. Unlike TFIID, USF alone was unable to prevent repression of the promoter during nucleosome assembly. Furthermore, USF displayed reduced or no transcriptional stimulatory activity when added to previously assembled minichromosomes. Under conditions of nucleosome assembly, USF increased the number of assembled minichromosomes which contained stable preinitiation complexes. Subsequent to assembly, the rate at which preformed complexes initiated transcription appeared to be independent of the presence of USF. Thus USF potentiated the subsequent transcriptional activity of the promoter indirectly, apparently by increasing the rate or stability of TFIID binding. This activity resulted in the promoter becoming resistant to nucleosome mediated repression. These observations suggest that some ubiquitous upstream factors, e.g. USF, may play an important role in establishing the transcriptional potential of cellular genes during chromatin assembly.

Key words: chromatin/MLTF/nucleosome/transcription/USF

Introduction

Chromatin structure plays an important role in the differential control of gene expression in eukaryotes. This is evidenced by the fact that tissue specific genes (e.g. globin) are transcribed when transfected into cells which do not express their endogenous chromosomal counterparts and are also transcribed *in vitro* as purified DNA when added to extracts from nonexpressing cells (reviewed in Weintraub, 1985). In yeast genetic alteration of the stoichiometry of core histones alters transcription patterns (Clark-Adams *et al.*, 1988) and the depletion of histone H4 leads to the activation of specific genes in the absence of the normal inducing agents (Kim *et al.*, 1988; Han *et al.*, 1988; Han and Grunstein, 1988). These studies illustrate that by suppressing basal levels of promoter activity nucleosomal structural proteins play a crucial role in transcription control.

In vitro functional studies have begun to reveal details of the relationship between nucleosome assembly and transcription initiation by RNA polymerase II. As shown previously for class III genes [reviewed in Brown (1984) and Wolffe and Brown (1988)] the assembly of the adenovirus-2 major late promoter into nucleosomes prevents subsequent transcription from the promoter by basal initiation factors and RNA polymerase II (Knezetic and Luse, 1986; Lorch *et al.*, 1987; Matsui, 1987; Workman and Roeder, 1987). However, if a stable preinitiation complex is formed on the promoter, prior to nucleosome assembly, the promoter remains active in subsequent transcription (Matsui, 1987; Workman and Roeder, 1987; Knezetic *et al.*, 1988). Furthermore, when the formation of preinitiation complexes is in direct competition with nucleosome assembly a transcriptional activator, the immediate-early protein of pseudorabies virus, stimulates preinitiation complex formation (Workman *et al.*, 1988). Thus, the potentiation of promoter function during chromatin assembly can be a regulatory event in transcriptional control.

In addition to RNA polymerase II, multiple basal transcription factors (termed TFIIA, TFIIB, TFIID and TFIIE) form a preinitiation complex at the TATA box and cap site (Nakajima *et al.*, 1988; Van Dyke *et al.*, 1988; Buratowski *et al.*, 1989) and are absolutely required for accurate transcription from the major late promoter (Matsui *et al.*, 1980; Samuels *et al.*, 1982; Sawadogo and Roeder, 1985a; Reinberg *et al.*, 1987; for a review of further fractionation of these proteins see Saltzman and Weinmann, 1989). Maximum *in vivo* and *in vitro* transcription from the major late promoter also requires a 43–50 kd cellular upstream transcription factor, USF (MLTF), which binds at approximately −58 relative to the cap site (Hen *et al.*, 1982; Miyamoto *et al.*, 1984; Yu and Manley, 1984; Carthew *et al.*, 1985; Miyamoto *et al.*, 1985; Sawadogo and Roeder, 1985b; Chodosh *et al.*, 1986; Moncollin *et al.*, 1986; Sawadogo, 1988; Sawadogo *et al.*, 1988). In order to investigate the function(s) of USF in a chromosomal context, we have used an *in vitro* 'chromatin' reconstitution system to examine the role of USF in the assembly and subsequent transcription of minichromosomes containing the major late promoter.

Results

The fold stimulation of transcription by USF is greater under conditions of nucleosome assembly

To address the effect of nucleosome assembly on transcriptional stimulation by USF we used a heat-treated supernatant prepared from *Xenopus* egg extracts as a source of nucleosome assembly factors. These assembly factors bind to exogenously added histones and will then transfer the histones to template DNA in the form of nucleosomes (Laskey *et al.*, 1978). When conducted in the presence of topoisomerases, nucleosome assembly is revealed in the

supercoiling of closed circular DNA (Laskey *et al.*, 1977, 1978; Glikin *et al.*, 1984). We have supplemented this *in vitro* nucleosome assembly system with mammalian basal transcription factors (TFIIA, TFIIB, TFIID, TFIIE and RNA polymerase II) (Matsui *et al.*, 1980; Sawadogo and Roeder, 1985a). Under these conditions the formation of preinitiation complexes on the major late promoter is in direct competition with the assembly of the promoters into nucleosomes (Workman *et al.*, 1988).

We employed a two step protocol in which a 1 h incubation for the assembly of preinitiation complexes and/or nucleosomes onto template DNA was followed by a 1 h transcription reaction as diagrammed in Figure 1A. In order to assay transcription from circular minichromosome templates we utilized a plasmid (pML-81C_2AT) which contains the major late promoter from position −81 to +10 (relative to the cap site) upstream of the G-less cassette (Sawadogo and Roeder, 1985a,b). Transcription of this template, in the absence of GTP, generates a 390 base transcript. In this assay, the addition of partially purified USF stimulated transcription in the absence of nucleosome assembly ~3-fold (Figure 1A, lanes 1 and 2), which is less than the 10-fold stimulation observed with an identically prepared USF fraction under standard transcription conditions (Sawadogo and Roeder, 1985b). When the assembly reactions were supplemented with increasing concentrations of histones, such that nucleosome assembly would occur (see below), transcription levels were decreased in all reactions (Figure 1A, lanes 3−8). However, repression of transcription at each histone concentration was more effective in the absence than in the presence of USF (Figure 1A, cf. lanes 1 and 2 with 3 and 4 or 5 and 6). Thus while both basal and stimulated levels of transcription were repressed by histone addition the observed fold stimulation of transcription resulting from the presence of USF (i.e. the fold increase over basal transcription at the same histone concentration) was greater when histones were present. As indicated in Figure 1A, quantitation revealed that fold stimulation of transcription by USF was maximal (15-fold) when 400 ng of histones were added to assembly reactions which contained 100 ng template DNA. In our separate experiments in which USF was assayed under increasing histone concentrations, the degree of stimulation varied but the maximum fold stimulation invariably occurred at 400 ng histones/reaction (Figure 1B).

The addition of purified histones to the assembly reactions resulted in the assembly of template DNA into nucleosomes (Figure 2A). Assembly reactions were identical to those in Figure 1A except for the use of internally labeled template DNA. Following the 1 h assembly reaction, the template topology was assayed on a 1% agarose gel. Lane 1 in Figure 2A shows that the input plasmid template contained moderately supercoiled closed circular DNA (form I), as well as nicked plasmid (form II) and a small amount of linear plasmid (form III). In the absence of histones the moderate supercoiled forms were relaxed during the 1 h assembly reaction (Figure 2A, lanes 2 and 3). The addition of histones resulted in the closed circular plasmid DNA becoming more negatively supercoiled, reflecting the assembly of the template into nucleosomes (Figure 2A, lanes 4−9). While nucleosome assembly proceeded under all of the histone concentrations tested, the template DNA was most efficiently assembled (i.e. driven into the most supercoiled forms) at

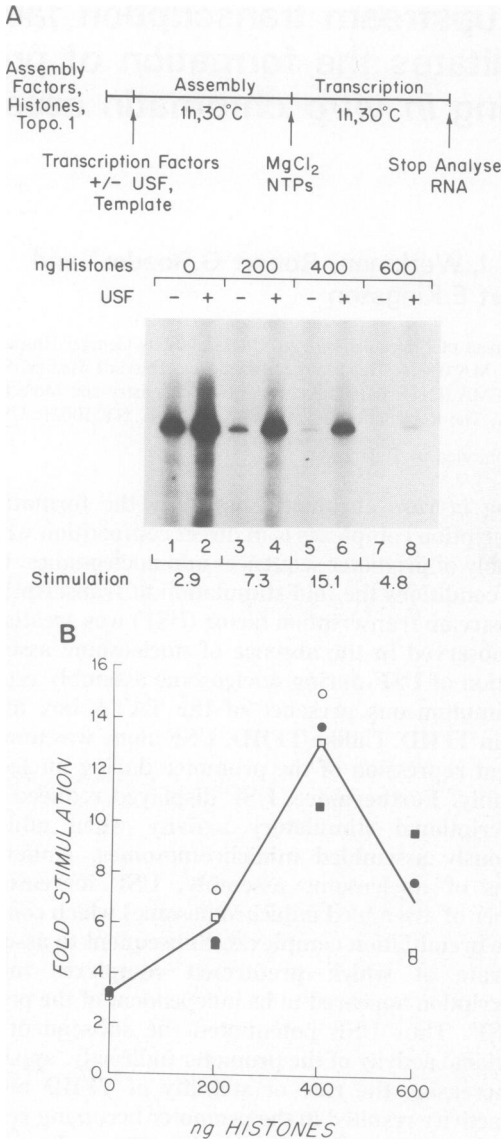

Fig. 1. The fold of transcription stimulation by USF is greater in the presence of histones. (**A**) RNA products synthesized from the major late promoter in the assembly/transcription assay diagrammed. Basal transcription factors, nucleosome assembly factors and template DNA were mixed at the start of the assembly step. USF and histones were added as indicated. See Materials and methods for experimental details. The observed fold stimulation in the samples containing USF is indicated for each histone concentration. (**B**) Four separate experiments in which the fold of transcriptional stimulation by USF was assayed with increasing histone concentrations are plotted. Each symbol represents the points of a separate experiment. (○) represents the experiment shown in (A). The line is drawn through the average value at each histone concentration.

400 ng of added histones (Figure 2A, cf. lanes 4, 6 and 8). The decrease in nucleosome assembly observed at 600 ng was presumably due to the concentration of added histones slightly exceeding the binding capacity of the acidic assembly factors present, as free histones inhibit both nucleosome assembly (see below) and transcription (Workman *et al.*, 1988).

The effects of histones on transcription and on template topology were due to the process of nucleosome assembly and not due to non-specific interactions of histones with DNA. The negative supercoiling of template DNA observed

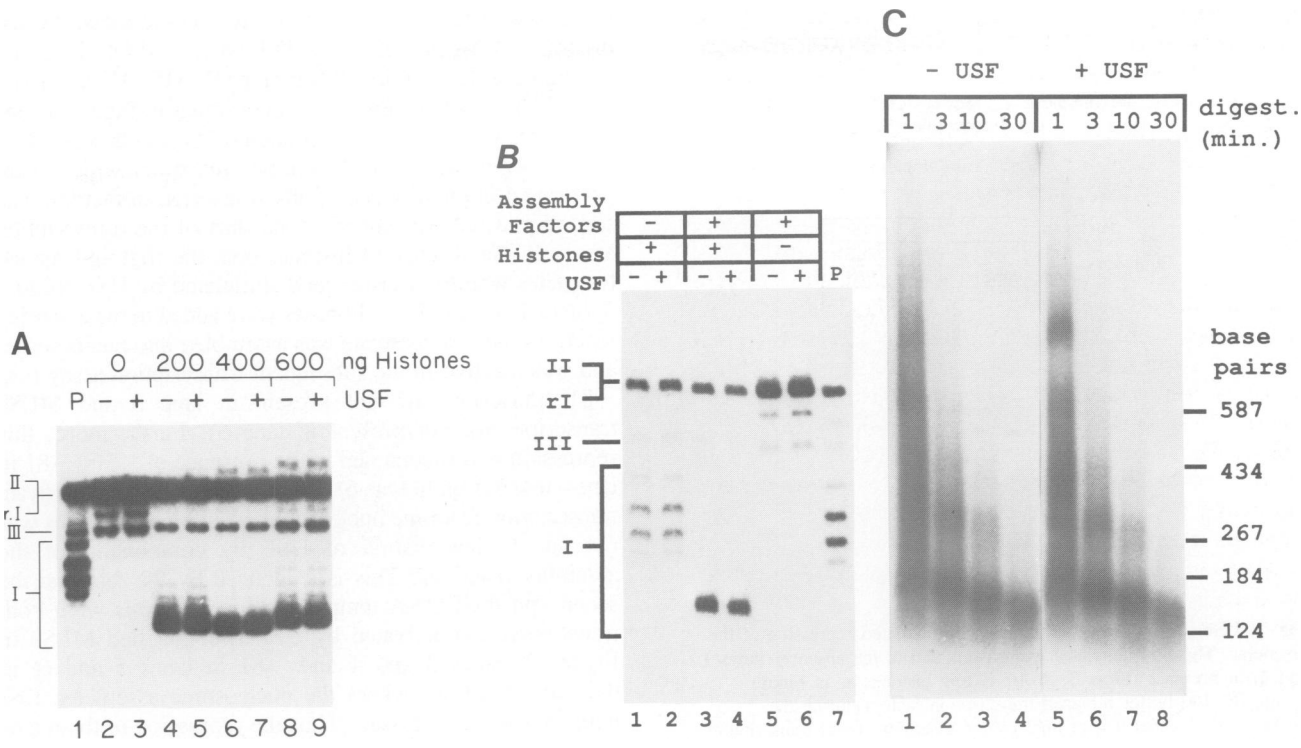

Fig. 2. The addition of histones results in the assembly of template DNA into nucleosomes. (**A**) Template supercoiling due to nucleosome assembly of template DNA. Assembly reactions were performed exactly as in Figure 1A except internally labeled template was used. Immediately following the assembly step the template topology was assayed as described in Materials and methods. The relative positions of nicked (II), linear (III), relaxed closed circular (r.I) and closed circular (I) plasmid are indicated. **Lane 1** shows the topology of the input plasmid. (**B**) Template supercoiling requires the presence of nucleosome assembly factors as well as histones. Template supercoiling during the assembly reactions (as diagrammed in Figure 1A) in the presence of both nucleosome assembly factors and 400 ng histones is shown in **lanes 3** and **4**. When histones were omitted template DNA was relaxed by topoisomerase 1 (**lanes 5** and **6**). When nucleosome assembly factors were omitted (and replaced by the corresponding buffer) the 400 ng of free histones precipitated template DNA such that neither relaxation nor nucleosome assembly occurred (**lanes 1** and **2**). USF was included or omitted during assembly as indicated. **Lane 7** shows the topology of the input plasmid. (**C**) Micrococcal nuclease digestion of assembled templates indicates that the template DNA was assembled into nucleosomes. Assembly reactions were performed with 400 ng histones (with or without USF) and were identical to those shown in lanes 3 and 4 of (B). Individual reactions were subsequently digested with micrococcal nuclease for the indicated times as described in Materials and methods. Shown is a 1.5% agarose gel of the digestion products. Note the appearance of a nucleosome size digestion product of ~180 bp (**lanes 1** and **5**) which was subsequently trimmed to ~145 bp (**lanes 4** and **8**). The positions of mol. wt markers run on this gel are indicated.

with addition of histones required the presence of the acidic assembly factors which transfer histones to DNA (Figure 2B, cf. lanes 3 and 4 with 5 and 6; also see Laskey *et al.*, 1978). When acidic assembly factors were omitted from assembly reactions the free histones precipitated template DNA, inhibiting both relaxation by topoisomerase 1 (Figure 2B, lanes 1 and 2) and the function of the template in subsequent transcription (Workman *et al.*, 1988). Thus the activity of histones in the assembly/transcription assay (i.e. template supercoiling and increasing the fold stimulation by USF; Figures 1A and 2B) required conditions in which the added histones function in the process of nucleosome assembly (reviewed in Laskey and Earnshaw, 1980).

Micrococcal nuclease digestions of template DNA after the nucleosome assembly reactions were used to characterize templates assembled in the presence of 400 ng histones (Figure 2C). A nuclease resistant product is detected at ~180 bp (Figure 2C, lanes 1 and 5) and was subsequently trimmed to ~145 bp (lanes 4 and 8) in good agreement with the digestion products of a mononucleosome and nucleosome core particle respectively (reviewed in Pederson *et al.*, 1986). Furthermore the appearance of the nucleosome size digestion product required both the presence of assembly factors and histones and was not observed when either of these components were omitted (data not shown; also see Workman *et al.*, 1988). Thus the detection of nucleosomes by micrococcal nuclease digestion correlates with the observed template supercoiling (Figure 2B). As described previously (reviewed in Laskey and Earnshaw, 1980) the reconstituted nucleosome assembly system did not result in homogeneous spacing of nucleosomes on the template DNA after the short (1 h) reaction. While the mononucleosome band first appears at ~180 bp (indicating that the average length of DNA/nucleosome is approximately physiological), a band indicative of close packed dinucleosomes is also present (i.e. at ~310 bp) and the dinucleosome region of the gel (i.e. from 300 to 400 bp) appears as a broad smear.

Note that the degree of nucleosome assembly as assayed by template supercoiling did not differ significantly between assembly reactions with or without USF (Figure 2A). Furthermore the nucleosome size micrococcal nuclease digestion products were observed after assembly in the presence or absence of USF (Figure 2C, cf. lanes 1–4 with 5–8). Thus the observed transcriptional stimulation by USF (Figure 1) was not due to USF inhibiting the activity of the assembly factors, histones or topoisomerase I in nucleosome assembly.

Taken together Figures 1 and 2 illustrate that maximum

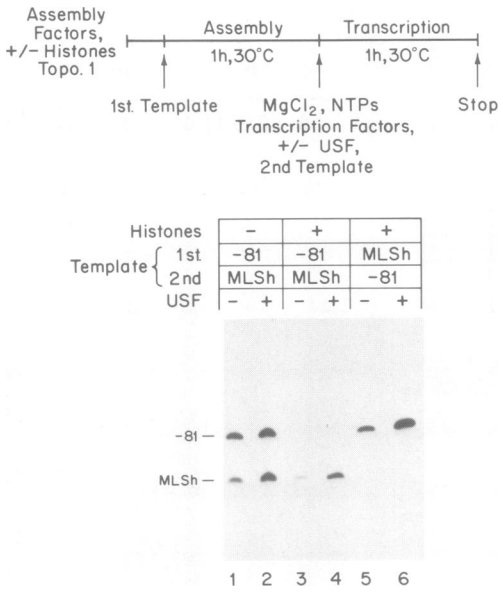

Fig. 3. USF does not reverse nucleosome mediated repression of the promoter. The first templates were assembled in the absence (**lanes 1** and **2**) or presence (**lanes 3–6**) of 400 ng histones as in Figure 1 except BC-100 buffer replaced transcription factors during the assembly step. After 1 h of nucleosome assembly, basal transcription factors, the second templates and USF (as indicated) were added followed by the transcription assay. The positions of transcripts generated from pML-81C_2AT (−81) and pMLSh (MLSh) are indiated.

fold stimulation of transcription by USF was achieved at the same histone concentration (400 ng/reaction) where nucleosome assembly was also maximal. This result indicates that USF effected a step in transcription which became rate limiting under conditions of nucleosome assembly. This agrees with an earlier report which indicated that the USF binding element decreased repression of the promoter under conditions of nucleosome assembly (Matsui, 1987). Addition of the USF fraction to assembly/transcription reactions containing a major late promoter construct deleted for the USF site (to −51; Sawadogo and Roeder, 1985b) did not result in stimulation of transcription (data not shown). Thus the observed stimulation required both the addition of the factor and the presence of the binding element on the promoter, indicating that the action of USF in the nucleosome assembly/transcription assay was mediated through the interaction of the factor directly with the promoter.

USF does not reverse or prevent nucleosome mediated repression of the promoter

The assembly of the major late promoter into nucleosomes has been shown to repress transcription from the promoter in nuclear extracts which contain USF (Knezetic and Luse, 1986; Matsui, 1987; Workman and Roeder, 1987). This suggests that nucleosome mediated repression is dominant over the action of USF. In order to more rigorously address this possibility, we tested the stability of nucleosome mediated repression in the presence of partially purified USF. To ensure that the protein remained active under conditions where minichromosomes were transcribed, we added a second purified DNA template in *trans*. In addition to pML-81C_2AT we used the plasmid pMLSh which contains the major late promoter from −400 to +10 (relative to the cap site) and thus also contains the USF binding site at −58. In addition, pMLSh contains a shortened G-less cassette and produces a transcript of ~350 bases, which is easily distinguishable from the 390 base pML-81C_2AT transcript (Van Dyke *et al.*, 1989). As diagrammed in Figure 3, one of two major late promoter containing plasmids was added at the beginning of the assembly reactions which were performed in the absence of any transcription factors. The second plasmid was added at the start of the transcription assay. In the absence of histones both the first and second templates were transcribed and stimulated by USF (Figure 3, lanes 1 and 2). When histones were added to the assembly reactions the first template was assembled into nucleosomes and was inactive in the subsequent transcription assay (i.e. −81 transcripts are not present in lane 3 and MLSh transcripts are not present in lane 5). Furthermore, this repression was maintained in the presence of USF (−81 in lane 4 and MLSh in lane 6). The inability of USF to activate transcription from the nucleosome assembled templates was not due to inactivation of USF by components of the assembly reactions. This is evidenced by the fact that the second purified DNA templates added in *trans* were both transcribed and activated by USF (i.e. purified MLSh in Figure 3, lanes 3 and 4 and −81 in lanes 5 and 6) in the same reactions where the nucleosome assembled first templates were repressed. Thus the repression of the major late promoter during the assembly reaction required histones, was mediated in *cis* and persisted in the presence of transcriptionally active USF. These results indicate that USF was unable to disrupt or alter pre-existing nucleosome structures in a manner leading to activation of the promoter.

The observed stimulation (Figure 1) could occur if USF binding prevented repression of the promoter by nucleosome assembly, as previously observed with the TATA box binding protein, TFIID (Workman and Roeder, 1987). To address this possibility, template DNA was preincubated with USF and/or additional transcription factors to allow factors to bind the promoter prior to nucleosome assembly (diagrammed in Figure 4). Using the reconstituted nucleosome assembly system, we found that preincubating template DNA with only the TFIID fraction resulted in suppression of transcription regardless of whether or not histones were added, indicating that inhibitory proteins co-purified with TFIID. This inhibition was alleviated when further proteins were present during preincubation. Thus the TFIIA fraction was included in all the preincubation reactions since this fraction contained the majority of the protein in the reconstituted transcription factors (see Materials and methods), thus normalizing the preincubation conditions, and did not show any ability to prevent promoter repression alone (Figure 4).

Figure 4 shows that after preincubation with TFIIA the promoter was repressed during nucleosome assembly (lane 1). After preincubation with TFIID and TFIIA or with all the basal factors some promoter activity remained after nucleosome assembly (Figure 4, lanes 3 and 5). In contrast, after preincubation with USF and TFIIA the promoter was repressed during nucleosome assembly (Figure 4, lane 2). Thus two sequence specific DNA binding proteins (USF and TFIID), which bind to adjacent sites on the promoter, differ with regard to their ability to prevent repression of the promoter during nucleosome assembly. However, when USF was present with either TFIID/TFIIA or a total complement of basal factors during the preincubation steps the subsequent

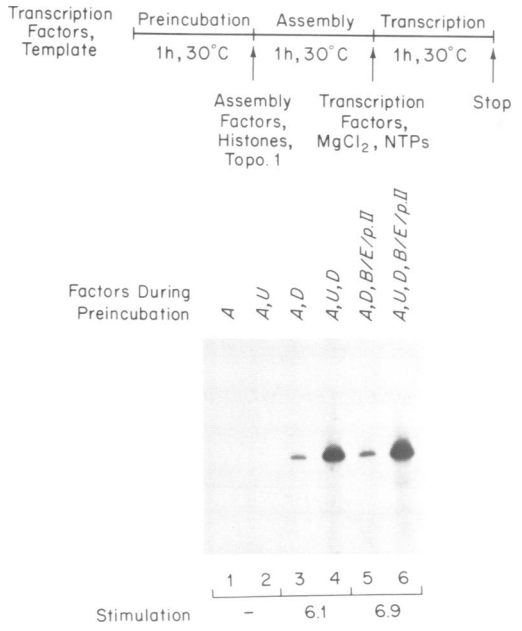

Fig. 4. USF activity during nucleosome assembly requires additional factors. Prior to the start of assembly reactions template DNA was preincubated with the transcription factors indicated to allow factor–promoter interactions without competition of nucleosome assembly (as diagrammed). Following assembly with 400 ng histones the remaining basal factors were added as necessary and transcription from the promoter was assayed. The fold of transcriptional stimulation resulting from the presence of USF is indicated for each condition of preincubation.

levels of transcription were stimulated (Figure 4, lanes 4 and 6). Thus the stimulatory effect of USF during nucleosome assembly requires the presence of basal initiation factors, minimally TFIID. This implies that either the number of preinitiation complexes resulting from the assembly step was increased in the presence of USF or that complexes containing USF were more active in transcription initiation.

USF increases the number of preinitiation complexes formed during minichromosome assembly

In order to determine if this stimulation by USF was due to a larger fraction of the assembled minichromosomes containing preinitiation complexes or to an enhanced initiation rate from complexes which include USF, we employed protocols which allow a measurement of the number of existing preinitiation complexes and the subsequent rate of initiation. The formation of preinitiation complexes is a slow step prior to the initiation of transcription. However, upon addition of nucleotide triphosphates, transcription initiation from pre-formed complexes is rapid (Hawley and Roeder, 1985, 1987). While preinitiation complexes are sensitive to low levels of Sarkosyl or moderate levels of KCl, once initiation occurs the resultant elongation complexes are stable in these conditions allowing transcription elongation to occur (Hawley and Roeder, 1985, 1987; Cai and Luse, 1987). When initiation is blocked by these reagents shortly after the addition of nucleotide substrates, transcription is limited to one round (reflecting the number of complexes) since the reassembly of complete preinitiation complexes is a slow step required prior to reinitiation (Hawley and Roeder, 1987).

Figure 5A shows an initiation time course from minichromosomes assembled in the presence of basal initiation factors with or without USF. After the assembly of templates into minichromosomes, the samples were brought into transcription conditions and nucleotide triphosphates were added. Subsequently, initiation was blocked by the addition of KCl to 200 mM (Cai and Luse, 1987) at various times followed by continued incubation to allow elongation. The rate of transcription initiation was detected by the increasing levels of the subsequently elongated transcripts (Figure 5A, lanes 1–7 and 8–14). As shown in Figure 5B transcription initiation occurred rapidly from minichromosomes assembled in either the presence or absence of USF and was essentially complete in a few minutes. Furthermore, the transcriptional stimulation observed from minichromosomes assembled in the presence of USF (~8-fold in this experiment) remained constant over the time course of initiation. Thus, after very short times of initiation the fold stimulation resulting from the presence of USF during assembly was the same as when initiation was not blocked (60 min time points). Together, these observations indicate that in both cases the observed initiation events were primarily first round events occurring from preinitiation complexes formed during the assembly step and that reinitiation did not contribute significantly to the observed transcription.

When the amount of initiation at each time point was plotted relative to the maximum amount of initiation that occurred in 60 min for each sample (as determined from Figure 5A, lanes 7 and 14), it is clear that the relative rate at which the preformed complexes initiated was essentially identical (Figure 5C). In fact, initiation in both cases resembled pseudo-first order kinetics with a $t_{1/2}$ of ~1 min. The initiation time course shown in Figure 5C is consistent with a logarithmic decay of previously formed preinitiation complexes (i.e. by initiation) which proceeded at the same rate regardless of the presence of USF. Thus, the stimulatory effect of USF during assembly was apparently due to formation of preinitiation complexes on an increased number of promoters rather than an increased rate of initiation. This observation is in agreement with the report of Carcamo *et al.* (1989) that USF increased the formation of rapid start complexes on DNA templates (containing the major late promoter) 2-fold during a preincubation step. In fact the 3-fold stimulation observed in the absence of histones (Figure 1) could reflect the same activity since under those conditions the assembly step consisted of a preincubation of the template with factors. However, when histones are added, nucleosome assembly effectively competes with preinitiation complex formation on the promoter, which becomes the limiting step in transcription (Workman *et al.*, 1988). Thus the activity of USF in facilitating the formation of preinitiation complexes resulted in a 2- to 3-fold stimulation on purified DNA templates and up to 15-fold stimulation under conditions of nucleosome assembly.

The conclusion that USF facilitated the formation of preinitiation complexes during nucleosome assembly suggests that for USF to function maximally in this assay it must be present from the onset of the assembly reactions. In the experiment shown in Figure 6 template DNA was preincubated under various conditions and USF was added either during the preincubation or after 1 h of nucleosome assembly. As shown above, preincubation with TFIID and TFIIA or all the basal factors preserved some promoter

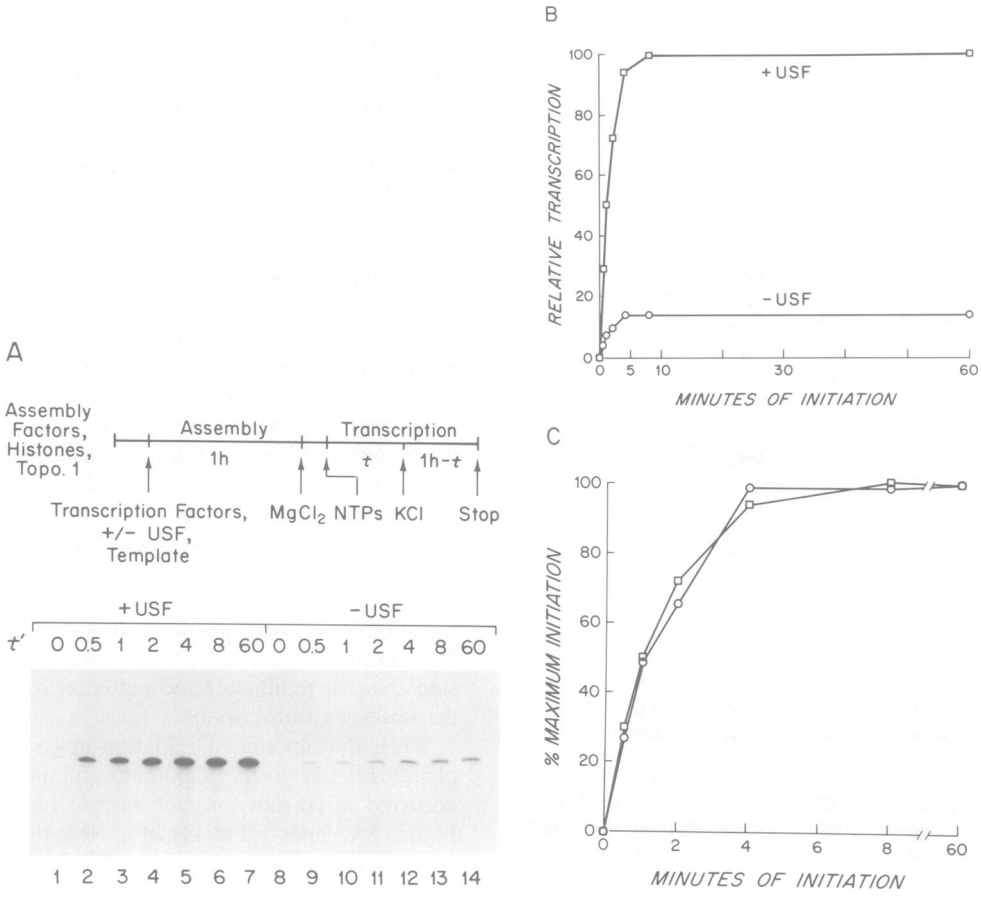

Fig. 5. USF increases the number of preinitiation complexes formed during nucleosome assembly. **(A)** Templates were assembled with 400 ng histones and all the basal initiation factors in the presence (**lanes 1–7**) or absence (**lanes 8–14**) of USF as in Figure 1A. Following template assembly the reactions were brought into transcription conditions and nucleotide triphosphates were added. Transcription initiation was blocked at the times indicated (by KCl addition to 200 mM) followed by continued incubation to allow elongation (see Materials and methods). The resultant transcripts are shown. Separate reactions were performed for each time point. **(B)** The relative levels of transcription from the experiment in (A) are plotted for reactions assembled in the presence (□) or absence (○) of USF. The amount of transcription observed without blocking initiation in the presence of USF (lane 7, in A) was set as 100, and the background levels of initiation (from the 0 points. lanes 1 and 8) were subtracted from each time point. The level of stimulation observed in the samples with USF remained constant (~8-fold) over all the time points. **(C)** The data shown in (B) are plotted as the percent of the maximum amount of initiation observed (60 min points) for samples assembled in the presence (□) or absence (○) of USF. In each case the transcripts generated are initiated rapidly (i.e. from preinitiation complexes formed during the assembly step) and at similar rates.

function (Figure 6, lanes 3 and 6) which was increased when USF was also present during template preincubation (lanes 5 and 8). In contrast, USF displayed no transcriptional stimulatory activity when added to minichromosomes previously assembled in the presence of all the basal factors (Figure 6, cf. lanes 6, 7 and 8). When added to minichromosomes previously assembled in the presence of TFIID and TFIIA, USF displayed a reduced but reproducible 2-fold stimulation of transcription (cf. lanes 3, 4 and 5). One possible explanation for the reduced transcriptional stimulation by USF after assembly is that nucleosomes may have blocked USF binding even on templates where preinitiation complexes occupy the promoter. To address this possibility, we performed the same set of experiments but simply omitted histones so that nucleosome assembly would not occur. These experiments demonstrated that the addition of USF to DNA templates preincubated with TFIID and TFIIA showed a similar reduced 2-fold stimulation and templates preincubated with all the basal factors were not stimulated at all (data not shown). Thus a similar reduction of stimulation was observed by the post-assembly addition of USF to minichromosomes or DNA templates containing preformed complexes.

Minichromosomes assembled in the presence of USF display enhanced accessibilty of a restriction endonuclease site adjacent to the promoter

Nucleosome free regions (hypersensitive sites), in cellular and viral chromatin, display enhanced accessibility to restriction endonucleases as well as nonsequence specific nucleases (reviewed in Gross and Garrard, 1988). We have utilized restriction endonuclease digestions to examine the accessibility of sites adjacent to the promoter (*Eco*RI at −87 on pML-81C_2AT, relative to the cap site) and distal from the promoter (*Hin*dIII at +425). In order to maximize promoter occupancy, pML-81C_2AT (which was internally labeled at the *Bam*HI site, +395, and recircularized) was reduced 10-fold while the total input DNA was kept constant (with previous experiments) by the addition of pUC13 (see Materials and methods). After template assembly the minichromosomes were digested with the first restriction endonuclease. The DNA was then purified and cut to

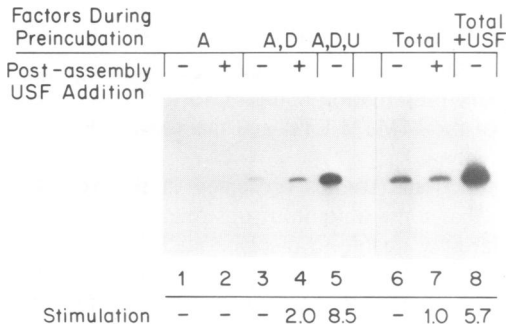

Fig. 6. Transcriptional activity of USF on preassembled templates. Template DNA was preincubated with the basal factors and USF as indicated prior to the addition of assembly factors and histones as in Figure 4. After 1 h of nucleosome assembly the remaining required basal factors and USF (where indicated) were added (see Materials and methods). After an additional 20 min incubation transcription from the templates was assayed. The fold stimulation resulting from the presence of USF (whether added before or after assembly) is indicated for each experimental condition.

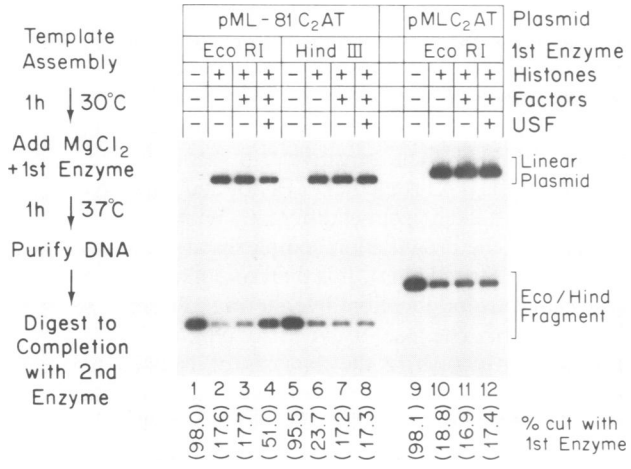

Fig. 7. Restriction endonuclease digestion of assembled minichromosomes. Following the assembly reactions, templates (either pML-81C_2AT or pMLC_2AT) were treated with the first enzyme, *Eco*RI or *Hin*dIII as indicated. The template DNA was purified and cut to completion with the second enzyme as diagrammed. Assembly reactions contained 400 ng histones, a complete complement of basal transcription factors, and USF as indicated. The locations of the linear templates resulting from minichromosomes which were refractory to digestion with the first enzyme and the *Eco*RI/*Hin*dIII fragments resulting from templates which were cut with the first enzyme are indicated. The percent of minichromosomes cut with the first enzyme is indicated for each digestion.

completion with the second enzyme (diagrammed in Figure 7) so that minichromosome digestion was revealed in the appearance of the labeled restriction fragment between the two restriction sites.

Figure 7 shows the result of restriction endonuclease digestions. The assembly of template DNA into minichromosomes (by the addition of histones to the assembly reactions) resulted in decreased cutting at either the promoter proximal *Eco*RI site or the distal *Hin*dIII site (Figure 7, cf. lanes 1 and 2; 5 and 6). Furthermore, the inclusion of basal initiation factors during template assembly did not significantly affect the accessibility of the *Hin*dIII site (Figure 7, lane 7) or the *Eco*RI site (lane 3). However, when USF was also included during the assembly reactions the resultant minichromosomes displayed enhanced accessibility (51% cleavage) of the promoter proximal *Eco*RI site (Figure 7, lane 4) but not at the distal *Hin*dIII site (lane 8). In addition when assembly reactions were performed with pMLC_2AT, which contains promoter sequences out to −404 such that the *Eco*RI site is located at −410 (Sawadogo and Roeder, 1985a), USF did not increase accessibility of the *Eco*RI site (Figure 7, lanes 9−12).

Thus, the inhibition of restriction endonuclease digestion resulting from nucleosome assembly was alleviated at a promoter proximal *Eco*RI site (at −87) but not at distal *Eco*RI (at −410) or *Hin*dIII (at +425) sites by the presence of USF and basal factors during nucleosome assembly. These results indicate that the action of USF resulted in structural perturbations in the promoter region of the template. Furthermore, the observation that a larger number of promoters remain accessible to *Eco*RI after template assembly in the presence of USF and basal factors is consistent with the earlier conclusion that an increased number of such promoters also contain preinitiation complexes. However, further structural analysis is necessary to determine if the *in vitro* binding of USF and/or preinitiation complexes actually results in the formation of a nucleosome free region over the promoter, which might resemble those observed *in vivo* [reviewed in Gross and Garrard (1988) and Elgin (1988)].

Discussion

The in vitro activity of USF in the assembly and transcription of minichromosome templates

Under conditions of *in vitro* chromatin assembly transcription is suppressed because nucleosome assembly effectively competes with basal transcription factors for occupancy of promoter sequences. Under these repressive conditions the fold stimulation of transcription observed by USF was greater than that observed during transcription of purified DNA templates. When present with basal initiation factors during nucleosome assembly, USF increased the number of assembled minichromosomes containing stable preinitiation complexes. However, the activity of USF during nucleosome assembly required minimally the simultaneous presence of TFIID (the TATA box binding protein); USF alone was unable to prevent repression of the promoter. Direct footprinting analysis has shown that promoter binding of both USF and TFIID is increased when both factors are present, presumably due to protein−protein interactions mediating cooperative binding (Sawadogo and Roeder, 1985b; Sawadogo, 1988). Cooperative binding could increase the rate of TFIID binding when USF was also present, such that during chromatin assembly a larger fraction of the promoters would sequester TFIID before becoming repressed by their assembly into nucleosomes. Stably bound TFIID would then render the promoter resistant to nucleosome mediated repression (Workman and Roeder, 1987). However, since the USF and the TFIID preparations used in our experiments were not purified to homogeneity, we cannot rule out the possibility that the effect of USF on TFIID binding was mediated through or required additional proteins which were present in these fractions.

The activity of USF during nucleosome assembly resembles that of the immediate early protein (IE) of pseudorabies virus. IE binds to multiple sites of weak

homology on the major late promoter (Cromlish *et al.*, 1989) and facilitates TFIID binding (Abmayr *et al.*, 1988), thus potentiating the promoter during nucleosome assembly (Workman *et al.*, 1988). Thus both IE and USF can prevent promoter repression indirectly via interactions with TFIID.

The addition of USF to previously assembled minichromosomes or to DNA templates which contained a complete preinitiation complex did not stimulate transcription. However, post-assembly addition of USF to templates containing only a committed complex (i.e. TFIID and TFIIA) (Davison *et al.*, 1983; Fire *et al.*, 1984; Reinberg *et al.*, 1987; Van Dyke *et al.*, 1989) resulted in a small but reproducible 2-fold stimulation of transcription. This observation suggests that in addition to facilitating TFIID binding, USF may increase the subsequent binding of TFIIB, TFIIE and/or RNA polymerase II. Such a two step activation mechanism has been suggested earlier by Sawadogo (1988) and is consistent with the observation that USF can stimulate reinitiation (Carcamo *et al.*, 1989) since polymerase and perhaps additional factors would have to recycle to the promoter where TFIID and USF remain stably bound (Van Dyke *et al.*, 1988).

Thus in a chromosomal context USF may provide two important activities. The first is to establish the transcriptional potential of the promoter during chromatin assembly (by facilitating the binding of TFIID) and the second to enhance the utilization of the promoter in transcription by increasing the loading of RNA polymerase II and additional factors. In this regard it is interesting that *in vivo* footprinting has revealed that USF is bound to the uninduced mouse metallothionein I promoter (Mueller *et al.*, 1988). Thus USF may participate in establishing the transcriptional potential of the metallothionen I promoter *in vivo* (which is fully realized upon metal induction) and may also provide for low levels of constitutive transcription.

Different functional roles of transcriptional regulatory proteins in the potentiation of gene activity in chromatin

The accurate regulation of eukaryotic genes often requires multiple regulatory proteins (for example, see Greene *et al.*, 1987) which may act at different potentially rate limiting steps. In a chromosomal context, perhaps the first such step is potentiating the activity of a promoter, in chromatin, by proteins which exclude nucleosomes during chromatin assembly or by proteins which can disrupt repressive chromatin structures [reviewed in Brown (1984) and Weintraub (1985); discussed in Workman *et al.* (1988)]. A large number of structural studies have shown that in fact such structural alteration precedes or accompanies the activation of individual genes *in vivo* [reviewed in Gross and Garrard (1988) and Elgin (1988)]. While the apparent mechanisms of formation and function of these accessible regions (nuclease hypersensitive sites) varies, inevitably the accessibility of the TATA box region to basal initiation factors and RNA polymerase II is achieved (see Elgin, 1988). For example, TATA binding proteins have been implicated directly in the formation of nucleosome free regions of *Drosophila* heat shock promoters into which the subsequent binding of heat shock factor stimulates transcription initiation and/or elongation (Wu, 1984; Rougvie and Lis, 1988; Thomas and Elgin, 1988). In contrast, disruption or displacement of nucleosomes by glucocorticoid receptor (Cordingley *et al.*, 1987; Richard-Foy and Hager, 1987; Perlmann and Wrange, 1988) or yeast PHO4 and/or PHO2 factors (Almer *et al.*, 1986; Han and Grunstein, 1988) apparently facilitates subsequent preinitiation complex formation on the TATA region of the MMTV LTR and the yeast PHO5 promoter respectively.

The functional studies presented in this report further indicate that some ubiquitous upstream factors, e.g. USF, are apparently unable to displace nucleosomes or directly prevent nucleosome occupancy of the promoter. However, such factors can potentiate promoters during chromatin assembly by facilitating binding of the TATA factor(s). Promoters containing binding elements for such upstream factors might more readily sequester TATA factor(s) after a localized disruption of chromatin structure or during chromatin assembly following DNA replication. In the latter case, the gene specificity of factors which function like USF may play a crucial role in determining which promoters will be potentiated during replication and thus accessible to RNA polymerases and basal initiation factors during the subsequent cell cycle. If the concentration of factors which are stable to nucleosome assembly (i.e. TFIID) becomes limiting during replication, upstream factors (e.g. USF) could determine which promoters are assembled into a potentiated conformation.

It is interesting to note that the *in vivo* activities of upstream factors differ in their sensitivity to the sequence of the corresponding TATA element (Taylor and Kingston, 1990) and thus most likely interact differently with the basal factors which form the preinitiation complexes at the TATA/CAP region (see Introduction). It is therefore likely that different upstream factors function by altering different steps in transcription. The use of *in vitro* chromatin assembly/transcription assays in the analysis of isolated upstream factors should allow an investigation into the individual roles (i.e. in potentiation, initiation and/or elongation) of multiple factors which bind a particular promoter. Such analysis may render valuable insights into the developmental and temporal control of gene transcription.

Materials and methods

Chromatin assembly reactions

Heat-treated *Xenopus* egg supernatants containing nucleosome assembly factors were prepared as described by Laskey *et al.* (1978). Histones were purified as a mixture (including H1, H2A, H2B, H3, H4 and H5) from isolated *Xenpus* erythrocyte nuclei as described (Workman *et al.*, 1988). Basal initiation factors were prepared from HeLa nuclear extracts according to standard protocols (Matsui *et al.*, 1980; Sawadogo and Roeder, 1985a). USF was isolated from HeLa nuclear extracts exactly as described by Sawadogo and Roeder (1985b).

Nucleosome assembly mixtures were prepared by incubating a mixture which consisted of 80% heat treated egg supernatant and 20% of the appropriate histone dilutions [dilutions were in TE (10 mM Tris, pH 8.0, 1 mM EDTA)] for 15 min at 20°C, to allow the histones to bind assembly factors, followed by the addition of topoisomerase 1 (Promega Biotec) to 0.24 U/μl. Ten microliters of the nucleosome assembly mixtures were mixed with 5 μl of reconstituted transcription factors. The composition of transcription factors added at the assembly step varied with different experiments (see figure legends); however, when present the amounts of particular fractions used were constant in all experiments. When fractions were omitted the volume was kept constant by the addition of BC-100, the buffer into which all the fractions were dialyzed. Five microliters of a total reconstitution of transcription factors (including USF) contained 5.3 μg protein of DEAE cellulose TFIIA fraction, 0.65 μg protein of DEAE cellulose TFIID fraction, 1.1 μg protein DEAE cellulose USF fraction, and

in the experiments shown, 3 μg protein of a phosphocellulose 0.5 M KCl step fraction which provided TFIIB, TFIIE and RNA polymerase II. The latter factors were simply added in one fraction since experiments adding isolated TFIIB, TFIIE and purified RNA polymerase II yielded similar results in the nucleosome assembly/transcription assay (data not shown; Workman *et al.*, 1988).

Assembly reactions were started by the addition of 100 ng template DNA (in TE) and proceeded for 60 min at 30°C. For experiments in which factors were preincubated with template DNA prior to nucleosome assembly, the 100 ng of template was incubated with 5 μl of various transcription factor mixtures in a total volume of 7 μl which also included 1.1 mM $MgCl_2$ (higher $MgCl_2$ concentrations inhibited subsequent topoisomerase I activity). After 6 min of preincubation at 30°C, 10 μl of the nucleosome assembly mixture was added and the assembly reactions proceeded as above.

Transcription reactions

Immediately following the assembly reactions transcription of the assembled templates was begun by the addition of 10 μl of TA [20% glycerol, 20 mM HEPES (pH 8.4), 15.5 mM $MgCl_2$, 1.5 mM ATP, 1.5 mM CTP, 0.25 mM 3′-*O*-methyl-GTP, 62 μmol [α-^{32}P]UTP (0.5 μCu/reaction), 1.5 U/μl RNase T1 (Pharmacia), 1.2 U/μl RNasin (Promega Biotec)]. Transcription was carried out for 60 min at 30°C after which the reactions were quenched and the RNA analyzed as described previously (Workman and Roeder, 1987). When transcription factors were added after template assembly all reactions received the same volume of factor(s) and/or BC-100 buffer which never exceeded 16% of the total volume of the transcription reactions. In the experiment shown in Figure 6, USF and additional factors were added after 1 h of nucleosome assembly; however, transcription was not started (by TA addition) until after an additional 20 min incubation at 30°C. This was done to test if USF binding might have been favored by the lower $MgCl_2$ concentration conditions in the assembly reactions (cf. Sawadogo, 1988). However,the results were identical to similar experiments where USF and TA were added simultaneously (data not shown). In the initiation rate experiment shown in Figure 5, TA without nucleotide triphosphates was added after the assembly reactions (to bring the samples into transcription conditions) and incubated an additional 10 min at 30°C. 1.4 M KCl (2.8 μl) was then added to the 0 time points (final KCl concentration 200 mM) followed by the addition of nucleotide triphosphates. Nucleotide triphosphates were added first to all other reactions and KCl was added to block initiation at the indicated times. All reactions were incubated for a total of 60 min at 30°C from the time of nucleotide addition.

Nucleosome assembly assays

Nucleosome assembly was assayed by the resultant supercoiling of plasmid pML-81C_2AT which was internally ^{32}P labeled at the *Bam*HI site (downstream of the G-less cassette) by the method of Razvi *et al.* (1983). Assembly reactions utilizing the labeled plasmid were performed exactly as described above except for the reactions shown in lanes 1 and 2 of Figure 2B, in which the heat treated egg supernatant was replaced by HA buffer (120 mM KCl, 2 mM $MgCl_2$, 20 mM Tris−HCl, pH 7.5), the buffer the egg extracts were prepared in. Immediately following the 1 h assembly reaction, samples were quenched by the addition of 5 μl 20% glycerol, 1.5% sodiumlaurylsulate, 70 mM EDTA (pH 7.5). One microliter of 10 mg/ml proteinase K was added followed by a 15 min incubation at 37°C. Samples were then loaded and run on 1% agarose TBE gels as previously described (Workman and Roeder, 1987). For the micrococcal nuclease digestions, $MgCl_2$ and $CaCl_2$ were added to 5 mM and 2 mM respectively, to separate samples for each time point immediately after the assembly reactions. One unit of micrococcal nuclease (Sigma) was added and the reactions were incubated at 20°C for the indicated times. Digestions were quenched with the SDS/EDTA stop mix used above, digested with proteinase K and run on 1.5% agarose TBE gels as previously described (Workman and Roeder, 1987).

Restriction endonuclease digestion of assembled minichromosomes

For restriction endonuclease digestion experiments the concentration of promoter containing plasmids (pMLC_2AT or pML81C_2AT internally labeled at the *Bam*HI site) was reduced to 10 ng/reaction. The total plasmid concentration was maintained at 100 ng/reaction by the addition of 90 ng pUC-13. Following the 1 h assembly reaction, $MgCl_2$ was added to a final concentration of 5 mM, and 6 U of *Hin*dIII or *Eco*RI (each at 5−12 U/μl; Boehringer Mannheim) was added as indicated in the legend to Figure 7. Digestion proceeded for 1 h at 37°C after which reactions were quenched by the addition of 100 μl of 100 mM NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA containing 10 μg/ml pUC-13 carrier DNA. The DNA was purified by extraction once with phenol and once with chloroform and precipitated with 250 μl of 95% ethanol, 100 mM sodium acetate. The DNA samples were resuspended in the appropriate restriction endonuclease buffer and digested to completion with the second enzyme (see Figure 7). Digestions were quenched with SDS/EDTA stop buffer used for supercoiling assays and loaded directly on 1.5% agarose TBE gels.

Analysis of RNA and DNA gels

Urea/acrylamide RNA gels and agarose DNA gels were dried and exposed to XAR film (Kodak). In addition all gels were counted and the bands quantitated using a Betascope 603 blot analyser (Betagen Corp.).

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