Regulated expression of the β -globin gene locus in synthetic nuclei

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Regulated gene expression within a complex chromosomal locus requires multiple nuclear processes. We have analyzed the transcriptional properties of the cloned chick β -globin gene family when assembled into synthetic nuclei made by use of *Xenopus* egg extracts. Assembly in an erythroid protein environment correctly recapitulates tissue-specific chromatin structure and long-range promoter–enhancer interaction within the chromosomal locus, resulting in β -globin gene activation. Nucleosome-repressed β -globin templates can be transcriptionally activated by double-stranded DNA replication in the presence of staged erythroid proteins by remodeling of the chromatin structure within the promoter region and establishment of distal promoter–enhancer communication. The programmed transcriptional state of a gene, as encoded by its chromatin structure and long-range promoter–enhancer interactions, is stable to nuclear decondensation and DNA replication unless active remodeling occurs in the presence of specific DNA-binding proteins.

[**Key Words:** β -Globin genes; transcription; enhancers; chromatin; *Xenopus*; DNA replication; synthetic nuclei]

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The structural organization of DNA within the eukaryotic nucleus is a critical determinant of transcriptional activity in a given cell type. Genomic DNA is initially assembled into nucleosomes and then into higher-order structures consisting of 10- to 30-nm filaments, which are arranged as 50- to 100-kb loop domains by attachment to the nuclear matrix or scaffold (Gasser and Laemmli 1987). DNA sequences found associated with the nuclear matrix (MARs or SARs) include regions that control transcription and DNA replication, such as enhancers, replication initiation sites, and boundary elements (Phi-Van and Stratling 1988). These observations and others suggest that gene regulation depends on a complex array of processes that require a precise nuclear architecture.

At the level of nucleosomal structure, active genes are characterized by a general nuclease sensitivity that extends over a large chromosomal domain, whereas inactive genes are packaged in very condensed chromatin that is resistant to nuclease cleavage (Wolffe 1990; Felsenfeld 1992; van Holde et al. 1992). These domains are often punctuated by nuclease hypersensitive regions that map to transcriptional control sequences and whose appearance is correlated with gene activation or repression (Weintraub 1985; Gross and Garrard 1988; Felsenfeld 1992). In vitro reconstitution studies have been particularly valuable in demonstrating that the interaction of specific DNA-binding proteins with promoter regions

can generate an altered, or accessible, chromatin structure that is transcriptionally derepressed. Although some proteins can interact with their DNA recognition sequences when incorporated into a nucleosome, many proteins can only bind to free DNA (Richard-Foy and Hager 1987; Perlmann and Wrangé 1988; Straka and Horz 1991; Taylor et al. 1991; Archer et al. 1992; McPherson et al. 1993). It has been postulated that DNA replication may be required for chromatin remodeling or activation of nucleosome-repressed genes by providing a brief opportunity for transcription factors to bind to DNA prior to chromatin reassembly (Wolffe and Brown 1986; Almouzni et al. 1990a; Wolffe 1991b). Paradoxically, once a pattern of gene expression is established in a given cell type, it must also be stable to transient disruption and reformation of chromatin structure during DNA replication. Thus, the generation and maintenance of a differentiated phenotype most likely depends on the concentration of activators or repressors present at the time that specific genes are replicated or become available for remodeling.

As an initial step in deciphering the complex nuclear processes involved in gene regulation, we have studied the transcriptional properties of cosmid containing the 38-kb chick β -globin gene locus when assembled into synthetic nuclei. These nuclei are formed by incubation of DNA with extracts from *Xenopus* eggs, which are a rich source of structural and enzymatic nuclear components (Almouzni and Wolffe 1993a). This in vitro system has been well-characterized for its ability to incorporate DNA into nuclear-like structures in discrete steps (Laskey et al. 1977; Lohka and Masui 1983; Newport 1987;

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Sheehan et al. 1988). Initially, DNA is assembled into chromatin, scaffold proteins then attach to nucleosomes and possibly induce condensation, and finally, membrane vesicles bind to this structure and fuse to form an intact double membrane, which is accompanied by chromatin decondensation (Newport 1987). After membrane formation is complete, a single round of semi-conservative DNA replication takes place utilizing a double-stranded DNA template (Blow and Laskey 1986, 1988; Newport 1987; Mills et al. 1989; Blow and Sleeman 1990; Cox and Laskey 1991; Leno and Laskey 1991; Hartl et al. 1994). These reconstituted nuclei are capable of active protein transport (Newmeyer et al. 1986a,b) and proceeding through the cell cycle (Murray and Kirschner 1989; Dasso and Newport 1990; Pfaller et al. 1991; Kornbluth et al. 1992). Thus, by several important criteria, these organelles resemble functional nuclei.

The chick β -globin chromosomal locus consists of four genes (5'- ρ - β^H - β^A - ϵ -3') that are expressed individually at defined stages of erythroid development (Choi and Engel 1988; Evans et al. 1990). Chromatin structural studies have demonstrated that the shared β^A - ϵ enhancer is nucleosome-free throughout erythroid development, whereas the promoter acquires an accessible conformation only at the onset of β^A -globin expression in definitive red blood cells (RBCs) (Stadler et al. 1980; McGhee et al. 1981; Reitman and Felsenfeld 1990). In vitro reconstitution experiments have shown that these stage-specific nucleosomal structures can be reproduced on cloned β^A -globin genes by use of RBC extracts or specific proteins from the appropriate time of development (Emerson and Felsenfeld 1984; Barton et al. 1993). In particular, two erythroid proteins, GATA-1 and NF-E4, can generate an active chromatin structure in the promoter and enhancer regions that result in efficient transcription from nucleosome-assembled β^A -globin templates (Barton et al. 1993).

In this study, we show that the β -globin chromosomal locus can be assembled into synthetic nuclei under conditions that precisely reproduce the erythroid-specific and developmentally regulated pattern of adult β^A -globin expression observed in definitive RBCs. These conditions are established by two different pathways: Stage-specific erythroid proteins must interact with the β -globin locus prior to chromatin formation or, alternatively, be present during DNA replication of nucleosome-repressed genes. In either pathway, transcriptional activation is dependent on a functional 3' β^A - ϵ enhancer, which must act at a distance of 2 kb on a nucleosome-free β^A -globin promoter. Our results indicate that DNA replication does not disrupt the transcriptional potential of a gene once it is defined by a specific chromatin structure. That is, a repressed template remains inactive and an active template regains its configuration on the newly synthesized daughter strands after replication. However, DNA replication does provide the opportunity for changing established transcriptional patterns by chromatin structural remodeling if the appropriate concentrations of activator proteins are available for binding. This has important implications for questions involving the

maintenance or plasticity of gene commitment as well as template switching within a multigene family. We hope that this in vitro reconstitution system will be generally useful for analyzing the mechanisms by which nuclear processes regulate the expression of natural genes within their proper chromosomal context.

Results

Incorporation of the chick β -globin chromosomal locus into synthetic nuclei

We have demonstrated previously that *Xenopus* egg cytosolic extracts are capable of assembling cloned chick β -adult globin genes into chromatin structures that are transcriptionally repressed in vitro. However, incubation of these genes with erythroid-specific DNA-binding proteins prior to nucleosome assembly generates chromatin templates that are poised for active transcription (Barton et al. 1993). Within the nucleus this type of chromatin modeling is an active process in which the binding of activator proteins must compete with chromatin assembly to determine the transcriptional state of a given gene. DNA replication during the cell cycle offers an opportunity for the interplay of core histones, nucleosome assembly factors, and transcriptional activator proteins. We wished to recreate this active process in vitro and to couple it with an assay to measure the transcriptional capabilities of nucleosome-assembled genes.

Previous work has shown that incubation of cloned DNA in *Xenopus* egg extracts, consisting of both soluble and vesicular components, leads to a stepwise assembly of chromatin structures that bind nuclear membrane vesicles prior to a single round of semiconservative DNA synthesis (Lohka and Masui 1984; Newport 1987; Blow and Laskey 1988; Lohka 1988; Sheehan et al. 1988; Mills et al. 1989; Blow and Sleeman 1990; Dasso and Newport 1990; Hartl et al. 1994). The processes of nuclear membrane formation and DNA replication are more efficient as the DNA template size increases (Blow and Laskey 1988). To recapitulate chromatin modeling as it occurs within the cell nucleus, including the formation of higher-order nuclear structures, we chose to study the chick adult β^A -globin gene within its normal chromosomal context. To this end, we cloned the entire β -type globin gene locus within a single cosmid (Barton et al. 1990), and focused on the chromatin assembly, replication, and transcription of this template (Fig. 1A).

The chick β -globin locus clone, sCos5 β A1, is efficiently reconstituted into synthetic nuclear structures when incubated with *Xenopus* egg extracts. Photomicrographs of the fluorescent-stained sCos5 β A1 DNA reveal that the process of nuclear assembly occurs in a stepwise, staged fashion and is accompanied by chromatin decondensation when bound to membrane vesicles (Fig. 1B, panels a–d,f). The fully assembled nuclear membrane, shown by phase-contrast microscopy, is present after 1 hr of incubation and is stable throughout the incubation period (Fig. 1B, panels e,g). The time course of DNA replication was also monitored during nuclear assembly by including [α - 32 P]dATP in the reaction. DNA

Figure 1. Reconstitution of the chick β -globin locus into synthetic nuclei. (A) Map of the β -globin gene locus clone. The cosmid clone, sCos5 β A1, encompassing the entire chick β -type globin locus has been described previously (Barton et al. 1990). The 38-kb insert is diagrammed with the coding regions of the embryonic (ϵ and ρ) and adult (β^H and β^A) stage-expressed genes shown as shaded boxes, and the shared β^A - ϵ globin enhancer as a small box. (B) Stages of nuclear reconstitution of the chick β -globin locus clone. One microgram of chick β -globin cosmid DNA was incubated with 100 μ l of a *Xenopus* egg extract as described. Aliquots were withdrawn at various times during incubation and analyzed by DNA fluorescent (*a-d,f*) and phase-contrast microscopy (*e,g*) and for DNA replication by incorporation of [α - 32 P]dATP into synthesized DNA, (*inset* graph). Incubation times: (*a*) 10 min; (*b*) 20 min; (*c*) 30 min; (*d,e*) 1 hr; and (*f,g*) 2 hr. Nuclear assembly is usually complete by 1–2 hr at 22°C and maximal DNA replication, assayed by incorporation of [α - 32 P]dATP into synthesized DNA, (*inset* graph), is observed within 3 hr.

polymerase α , present in the *Xenopus* egg extracts, incorporates the labeled substrate into the growing strands of DNA by de novo synthesis. By comparing the time course of [α - 32 P]dATP incorporation into DNA and the stages of nuclear assembly within the same reaction (Fig. 1B, *inset* graph), one can see that replication of double-stranded DNA in *Xenopus* egg extracts depends on the formation of nuclear membrane structures.

In our studies, we wished to examine the transcriptional properties of chromatin-assembled β -globin gene loci both before and after DNA replication. To achieve this, *Xenopus* egg extracts were fractionated by centrifugation into soluble and vesicular components. The *Xenopus* egg fractionation method has been well-characterized in several laboratories in terms of nuclear formation and subsequent DNA replication once the soluble and vesicular fractions are combined with DNA (Newport 1987; Sheehan et al. 1988; Leno and Laskey 1991; Hartl et al. 1994). The soluble fraction assembles physiologically spaced histone octamers (Rhodes and Laskey 1989) and is the same source of histone and nucleosome assembly factors that we employed previously to analyze transcription of β^A -globin chromatin templates in vitro (Barton et al. 1993).

DNA replication of the β -globin chromosomal locus in synthetic nuclei

Under our established conditions for chromatin assembly preceding transcription, the *Xenopus* egg extract comprises only 50% by volume of the chromatin assembly reaction before addition of in vitro transcription

components. Addition of the membrane vesicles under these conditions results in efficient DNA synthesis as monitored by incorporation of [α - 32 P]dATP over time and analysis by agarose gel electrophoresis (Fig. 2A, lanes 1–5). Duplicates of these reactions were incubated under the same conditions but in the presence of a DNA polymerase α inhibitor, aphidicolin. This drug also strongly effects the activity of eukaryotic DNA polymerases, δ and ϵ . Inclusion of aphidicolin completely abolishes the DNA replication that occurs on addition of membrane vesicles and formation of a nuclear membrane structure (lanes 6–10). 32 P-Labeled DNA replication reactions separated on nondenaturing agarose gels appear to migrate as a single band of supercoiled DNA. Extended exposure of these gels reveals no smears or shorter intermediates of replicated DNA (data not shown). Thus, the observed DNA synthesis is not the result of a gap-repair mechanism or single-stranded DNA replication, in agreement with previous results (Newport 1987).

The time course of replication is consistent with the assembly of nuclear membrane structures onto preformed chromatin, which precedes DNA replication (Fig. 1B). The appearance of a plateau of maximal DNA synthesis from 2–4 hr agrees well with previously published studies of synthetic nuclei assembly in two stages with a fractionated *Xenopus* egg system (Newport 1987; Sheehan et al. 1988; Mills et al. 1989; Leno and Laskey 1991; Hartl et al. 1994). The amount of input DNA replicated under these conditions (60–70%) was determined in several studies by measurement of the incorporation of [α - 32 P]dATP into high molecular weight DNA over the 2 hr incubation period (Blow and Laskey 1986).

Figure 2. Replication of the chromatin-assembled β -globin gene locus is dependent on the addition of *Xenopus* egg membranes. (A) Time course of replication: After preincubation and chromatin assembly of a cosmid containing the chick β -globin locus (0.5 μ g of sCos5 β A1) as described, the membrane vesicle fraction of a *Xenopus* egg extract and 1 μ Ci of [α - 32 P]dATP were added at time = 0. Continuous labeling of newly replicated DNA was monitored by removal of aliquots at times of 0, 30 min, 1 hr, 2 hr, and 4 hr. These samples were processed and analyzed by separation on a 0.8% agarose gel (lanes 1–5). Parallel samples were incubated under these exact conditions with the addition of 50 μ g/ml of aphidicolin (lanes 6–10). (B) Extent of replication: The chick β -globin gene locus (0.5 μ g of sCos5 β A1) was digested with *Eco*RI, *Eco*RI–*Hind*III, *Hind*III, *Hind*III–*Bam*HI, and *Bam*HI restriction endonucleases (lanes 1–5, respectively), analyzed by gel electrophoresis, and blotted onto a Gene-Screen Plus membrane. 32 P-Labeled newly replicated DNA was synthesized as described (0.5- μ g reaction) and used as a hybridization probe to determine the efficiency and extent of replication. The restriction enzyme-digested fragments present in the sCos5 β A1 clone are shown in reference to λ *Hind*III standards. (C) Chromatin structure of replicated DNA: Chick β -globin cosmid DNA (0.5 μ g) was nucleosome-assembled and replicated in *Xenopus* egg extract soluble and vesicular fractions as described. Nuclei-assembled DNA was digested with staphylococcal nuclease, and one-tenth volume aliquots were withdrawn for analysis at the following times: 0, 2.5, 5, 10, 20, 40, and 80 min (lanes 1–7, respectively). Nucleosome spacing was estimated by comparison to a 123-bp DNA ladder (lane M; Gibco-BRL). (D) Mononucleosome analysis of replicated, unreplicated, and total DNA: Staphylococcal nuclease limit digests were performed on sCos5 β A1 DNA (0.5 μ g) preincubated in buffer or 11-day RBC proteins followed by chromatin assembly for 1 hr (lanes 1,2) or nuclei formation with coupled chromatin assembly and 2 hr replication after membrane addition (lanes 3–6). The resulting mononucleosomes were separated by electrophoresis on a 1.8% agarose gel, blotted onto a Gene-Screen Plus membrane, and probed with 32 P-random-primed labeled chick β^A -globin DNA (lanes 1–4). Newly synthesized DNA in parallel reactions was labeled by the inclusion of 1 μ Ci of [α - 32 P]dATP during the replication period and analyzed as described above. Nucleosome assembly of the replicated 32 P-labeled β -globin locus DNA was demonstrated by exposure of the blotted agarose gel to film (lanes 5,6).

We next examined the extent of DNA synthesis within the cosmid containing the β -globin locus by utilizing the newly synthesized, 32 P-labeled DNA as a hybridization probe of the restriction enzyme-digested β -globin cosmid in a Southern blot analysis (Fig. 2B). All of the predicted restriction fragments from the sCos5 β A1 cosmid hybridized to the 32 P-labeled DNA synthesized de novo within chick β -globin synthetic nuclei assembled as described above. The hybridization

pattern is equivalent to the size and number of the restriction fragments observed in the ethidium bromide-stained agarose gel (data not shown) indicating that DNA synthesis appears to be uniform throughout the entire β -globin locus in this in vitro system.

The chromatin structure of newly replicated DNA in synthetic nuclei was assessed by performance of a time course of digestion with staphylococcal nuclease (Fig. 2C). After digestion, newly synthesized 32 P-labeled DNA

was purified and then separated by agarose gel electrophoresis prior to autoradiography. In this experiment, a regular array of nucleosomes with a spacing of ~ 180 bp is observed on replicated β -globin cosmid DNA (lanes 2–7), which is identical to that obtained from unreplicated DNA when assembled into synthetic nuclei (data not shown). Thus, DNA synthesized *de novo* in this system is rapidly assembled (within 15 min, data not shown) into chromatin structures similar to those found *in vivo*, and the bulk nucleosomal structure of replicated DNA is indistinguishable from that of unreplicated chromatin. A further characterization was made by quantitation of the mononucleosomes present on β -globin cosmid DNA in synthetic nuclei on limit staphylococcal nuclease digestion (Fig. 2D). This analysis reveals that similar amounts of nucleosomes are formed before (lanes 1–2) and after (lanes 3–4) replication when measured on bulk DNA or on newly synthesized DNA alone (lanes 5–6). Moreover, the presence of erythroid nuclear proteins in synthetic nuclei appears to slightly increase the number of nucleosomes assembled on both the replicated and unreplicated β -globin chromosomal locus (lanes 2,4,6). β -Globin cosmids deleted of the 3' β - ϵ enhancer are replicated in an identical manner to that of cosmids containing the intact locus by the criteria used in these studies (data not shown). This indicates that the enhancer has little influence on the extent and efficiency of DNA synthesis in this *Xenopus* system and does not function as a replication origin under the conditions employed.

Transcriptional activation of chromatin repressed β -globin genes requires DNA replication

Once conditions were established for the formation of transcriptionally active β^A -globin chromatin with *Xeno-*

pus egg cytoplasmic extracts (Barton et al. 1993) and, separately, the replication of double-stranded DNA by the addition of membrane components, we wished to combine the two reactions in a coupled assay to evaluate the potential for *in vitro* chromatin remodeling and transcription in this synthetic nuclei system. The protocol for these assays is diagrammed in Figure 3.

The β -globin chromosomal locus was initially assembled into nucleosomes in the absence [odd-numbered lanes, RBC^{pre} (–)] or presence [even-numbered lanes, RBC^{pre} (+)] of 11-day erythroid nuclear proteins (Fig. 4A). In the absence of the membrane fraction, erythroid proteins must be present prior to chromatin assembly to form transcriptionally active β^A -globin templates (lanes 2,8) because proteins added postassembly (lane 7) or omitted (lane 1) fail to activate nucleosome-repressed genes, as demonstrated previously with the isolated β^A -gene (Barton et al. 1993). β^A -Globin activation is observed only in synthetic nuclei formed in the presence of stage-specific erythroid proteins from early definitive RBCs (11-day), the stage in which the endogenous gene is maximally expressed. Protein extracts from primitive RBCs (4-day) or chick brain (11-day) fail to generate transcriptionally active β^A -globin genes (data not shown). Repressed β^A -globin templates remain inaccessible to 11-day RBC proteins within the *in vitro* transcription extract added after the completion of DNA replication (lanes 3,5), whereas transcriptionally activated genes remain active and are not converted to silent chromatin on decondensation of DNA during a single round of DNA synthesis (lanes 4,6). Thus, the established program of gene expression that is inherent in specific chromatin structures appears to be stable to DNA decondensation and replication in synthetic nuclei.

When DNA replication occurs in the presence of erythroid proteins [RBC^{repl} (+)], a dramatic change in β^A -

Figure 3. Diagram of the coupled chromatin assembly, replication, and transcription assay. Cosmid DNA encompassing the chick β -globin gene locus was assembled into nucleosomes by incubation in a *Xenopus* egg cytoplasmic soluble extract, after exposure to RBC nuclear proteins (RBC^{pre}) or buffer only. Chromatin-assembled DNA templates were either transcribed immediately on addition of NTPs and an RNA polymerase II-dependent erythroid transcription extract (upper pathway), or reconstituted into synthetic nuclear structures by addition of a *Xenopus* egg membrane vesicle fraction (lower pathway). Formation of an intact nuclear membrane around the chromatin template precedes the initiation of a single round of double-stranded DNA replication. The newly synthesized DNA is rapidly assembled into chromatin in the presence of RBC extract (RBC^{repl}) or buffer. These synthetic nuclei templates are then transcribed *in vitro* as described and analyzed for RNA synthesis.

Figure 4. (A) Transcription of the chromatin-assembled β -globin gene locus when incorporated into synthetic nuclei. Transcriptionally repressed β -globin chromatin templates can be activated by the addition of erythroid proteins and nuclear membranes. During the preincubation period, designated RBC^{pre}, 0.5 μ g of chick β -globin locus DNA was incubated with buffer only [odd-numbered lanes, RBC^{pre} (-)], or 11-day RBC nuclear extract [even-numbered lanes, RBC^{pre} (+)]. Following chromatin assembly by addition of *Xenopus* egg cytoplasmic fraction (20 μ l, 50 mg/ml) to each sample, all reactions were incubated in the absence (lanes 1,2,7,8) or in the presence (lanes 3–6, 9–12) of the membrane vesicle fraction as described. Buffer only (lanes 1–6) or 11-day RBC extract (lanes 7–12) was introduced during the replication incubation period, designated RBC^{repl}. Amounts of 11-day RBC nuclear extract employed in the reactions are RBC^{pre}, 20 μ l; RBC^{repl}, 10 μ l. Four microliters of a membrane vesicle fraction (15 mg/ml protein concentration) was added during the RBC^{repl} incubation step as designated (+). (B) Quantitation of β^A -globin transcription levels in synthetic nuclei when activated by DNA replication-dependent or -independent pathways. Chromatin and synthetic nuclei assembly reactions were exactly as described for the reactions shown in A. The 11-day RBC nuclear extract was added during the preincubation or replication steps as indicated (+) (RBC^{pre} = 20 μ l; RBC^{repl} = 10 μ l). Membrane vesicles were added as described previously to all reactions. pRSVcat plasmid DNA (100 ng) was added during the final 1-hr transcription period only. All transcription reactions were divided into halves, with one half processed by S1 analysis for the β^A -globin RNA transcript signals and the other half analyzed by primer extension of RNA from a synthetic CAT oligonucleotide for the RSV promoter-driven CAT RNA transcripts. Autoradiographs of the respective analyses are displayed with the equivalent reactions aligned vertically. The autoradiographs were scanned on a flatbed HP Laser Scanner and the images acquired by the DeskScan II program application. After importation into the NIH Image 1.44 analysis program, transcription signals were quantified and compared. Analysis and comparison of the duplicate reactions for transcription activation by preincubation, DNA replication remodeling, or both, reveals the following:

Activation pathway	Area units	Units/ μ l of RBC	Fold activity	Fold/ μ l of RBC
Preincubation	1263	63	9.5	0.5
Replication	588	51	4.0	0.4
Both	1521	59	18.4	0.6

These data indicate that the two pathways of transcriptional activation are equivalent, with the preincubation pathway being somewhat more efficient.

globin expression is observed. On addition of membrane vesicles, initiation of DNA replication and potential remodeling of chromatin structure occur in the presence of 11-day RBC nuclear proteins to generate β^A -globin genes that are highly expressed (lanes 9,11) when compared to repressed nucleosomal templates (lane 1) or templates incubated separately with membranes (lanes 3 and 5) or erythroid proteins (lane 7). These results establish a re-

quirement for erythroid activator proteins at critical stages of chromatin assembly to generate templates that are efficiently transcribed. The activation process can be achieved in vitro by reconstitution of chromatin around pre-existing protein–DNA complexes or by establishment of these complexes on repressed chromatin during DNA replication. If the precise proteins necessary to generate active chromatin on a specific gene are not

present in sufficiently high concentrations during DNA replication, then an inactive nucleosomal structure will presumably re-form, and the gene will remain transcriptionally inactive.

In these experiments, the RNA signal produced by transcription of β^A -globin chromatin is proportional to the amount of erythroid activator proteins present during preincubation (Fig. 4A; Barton et al. 1993). For example, the concentration of erythroid proteins during DNA replication of repressed chromatin, (Fig. 4A, lanes 9,11), is 50% of that used to activate the β^A -globin gene when added prior to chromatin assembly (lanes 2,4,6). Although the total amount of erythroid proteins present in the entire process may be equivalent, only β -globin gene loci that undergo replication by the addition of membrane vesicles show any increase in expressed β^A -globin RNA (cf. lane 8 with lanes 10 and 12). The ratio of RNA synthesized to the concentration of erythroid proteins is approximately equivalent in both pathways of activation. We have confirmed this (Fig. 4B) by quantifying β^A -globin transcription levels in the presence of an internal control (pRSV-CAT) in an experiment similar to that shown in Figure 4A. These data (Fig. 4A,B) support the conclusion that, in terms of the transcription potential of β^A -globin genes, the assembly of chromatin on replicated or unreplicated DNA appears to occur by a similar process in this *in vitro* system. Although the changes in absolute amounts of RNA produced *in vitro* are comparable to the concentration of activators present during chromatin structure formation or reformation, such comparisons of small changes (two- to threefold) in transcriptional activity, are not always reproducible in crude extracts [M.C. Barton, unpubl.].

An inhibitor of DNA polymerase abolishes replication-dependent chromatin activation

The experiments in the preceding section demonstrate that addition of membrane vesicular components to chromatin-assembled templates can have a significant effect on gene expression. To confirm that this is due to replication, we tested whether the eukaryotic DNA polymerase inhibitor, aphidicolin, could specifically abolish the ability of the membrane fraction to initiate active transcription from chromatin-repressed β^A -globin genes. As shown previously, this inhibitor efficiently prevents replication of β -globin gene loci in synthetic nuclei under the exact incubation conditions used in our transcription experiments (Fig. 2A).

Transcription of chromatin-repressed genes that are activated by the membrane fraction in the presence of erythroid proteins (Fig. 5, lanes 5,7) is completely abolished by the addition of aphidicolin (lane 9). As expected, β^A -globin templates that are generated by incubation with erythroid proteins prior to chromatin assembly (lanes 4,10,14) remain transcriptionally competent in the presence of aphidicolin because their activated nucleosomal structure is not dependent on DNA replication. This experiment substantiates the observation that DNA replication can neither activate chromatin-re-

Figure 5. Inhibition of DNA replication in synthetic nuclei prevents the activation of transcriptionally repressed β^A -globin chromatin templates. The DNA polymerase inhibitor aphidicolin was included in lanes 3,4,9,10,13, and 14 to assess the role of replication in the activation of chromatin during incubation with membranes and 11-day RBC proteins. Chromatin assembly and RBC^{pre} incubation conditions were as described. The membrane vesicle fraction was added to lanes 5–14 of which replication can occur only in reactions minus aphidicolin (lanes 5–8,11,12). Transcriptional activation of repressed chromatin structures that were not preincubated with erythroid proteins [RBC^{pre}(–), odd-numbered lanes] resulted only when the β -globin locus DNA was replicated after the addition of membranes in the presence of 11-day erythroid extracts [RBC^{rep1}(+), 10 μ l of 11-day RBC extract, lanes 5,7]. Inhibition of DNA replication under these conditions prevents transcriptional activation of repressed templates (lane 9). Identical concentrations of membrane fractions were added as in Fig. 4A.

pressed β^A -globin genes in the absence of erythroid proteins (lane 11) nor can it erase transcriptionally competent nucleosomal configurations (lane 12), confirming the results shown previously (Fig. 4A,B). We interpret our findings to indicate that DNA replication provides a window of opportunity in which genes packaged into inactive chromatin can be remodeled by interaction with activator proteins that are available at that time. These proteins influence the positioning of nucleosomes assembled after replication and thereby alter the transcriptional potential of the gene.

Chromatin remodeling by erythroid proteins during DNA replication accompanies gene activation

Evidence for specific changes in the chromatin structure of repressed β^A -globin genes that have been activated in a DNA replication-dependent process is provided by restriction enzyme accessibility studies of the promoter region. This region undergoes a pronounced structural change as a result of the loss of a nucleosome at a precise time in chick erythroid development (early definitive RBC) when β^A -globin genes are expressed [McGhee et al. 1981]. Chromatin reconstitution experiments have

shown that this nuclease hypersensitive structure can be generated *in vitro* by the interaction of specific DNA-binding proteins from definitive RBC extracts with cloned β^A -globin genes prior to nucleosome assembly (Emerson and Felsenfeld 1984; Barton et al. 1993). In these assays nuclease hypersensitivity is measured by the ability of the restriction enzyme *MspI* to cleave a 115-bp fragment from the β^A -globin promoter when this region is nucleosome free in both definitive erythrocytes or reconstituted chromatin.

In the present analysis, β -globin chromosomal loci were assembled into synthetic nuclei under conditions in which β^A -globin genes are active or repressed and then digested with *MspI* to monitor specific changes in promoter accessibility. As shown in Figure 6, there is a clear correlation between an open chromatin structure, as indicated by the release of the 115-bp fragment from the

Figure 6. Transcriptional activation of repressed β^A -globin genes is the result of chromatin structural remodeling during replication. *MspI* cleavage sensitivity of the β^A -globin promoter region in reconstituted synthetic nuclei measured by the specific release of an 115-bp fragment ($\rightarrow$), was assayed as described by probing with a fragment of the β^A -globin promoter spanning the *MspI* sites at -224 to -109 . (Lane 1) The *MspI* digestion of nucleosome-free β -globin cosmid DNA; (lanes 2–5) the *MspI* digestion of DNA reconstituted into synthetic nuclei by incubation with *Xenopus* egg extract soluble plus vesicular fractions as follows: (Lane 2) Preincubation and replication in buffer only [RBC^{pre}(–); RBC^{repl}(–)]; (lane 3) Preincubation in buffer only and replication in the presence of 20 μ l of an 11-day RBC nuclear extract [RBC^{pre}(–); RBC^{repl}(+)]]; (lane 4) preincubation with 20 μ l of an 11-day RBC nuclear extract and replication in buffer [RBC^{pre}(+); RBC^{repl}(–)]; (lane 5) preincubation with 20 μ l of an 11-day RBC extract and replication in the presence of an additional 20 μ l of an 11-day RBC extract [RBC^{pre}(+); RBC^{repl}(+)].

β^A -globin promoter, and transcriptional activity. For example, β^A -globin genes assembled into synthetic nuclei in the absence of erythroid proteins are transcriptionally repressed and display no promoter accessibility (lane 2) relative to free DNA (lane 1). By contrast, genes assembled into transcriptionally active synthetic nuclei by either preincubation with RBC factors (lane 4) or incubation with these factors during DNA replication (lane 3) or both (lane 5) have greatly increased promoter sensitivity to *MspI* cleavage. This clearly indicates that the loss of a nucleosome from the β^A -globin promoter is a major structural feature of active chromatin. This open promoter configuration can be formed by two different pathways to generate active chromatin, replication-dependent (lane 3) or independent (Emerson and Felsenfeld 1984; Barton et al. 1993). Once established, the open promoter is stable to DNA replication because all chromatin templates in this experiment were incubated with the membrane fraction (lanes 2–5). This is consistent with the observation that the transcriptional potential encoded by specific chromatin structures remains unchanged after DNA replication unless conditions favor template remodeling (Figs. 4A,B and 5).

Transcription of the β^A -globin gene in synthetic nuclei requires the action of a distal enhancer

The tissue-specific and developmentally regulated expression of the β^A -globin gene is completely dependent on a distal enhancer element residing 2 kb 3' of the structural gene (Hesse et al. 1986; Choi and Engel 1988). However, attempts to reproduce this and other distal enhancer effects *in vitro* with natural genes as templates have been largely unsuccessful. Recent experiments with model genes have shown that long-range activation by GAL4–VP16 is achieved only when the templates are assembled into histone H1-containing chromatin (Laybourn and Kadonaga 1992). We wished to examine the enhancer dependence of β^A -globin expression in synthetic nuclei to determine how accurately this system can reproduce the major aspects of tissue-specific regulation observed *in vivo* and to assess its potential as a valid *in vitro* approach to decipher the mechanisms involved in long-range transcriptional control.

As shown in Figure 7, efficient expression of the β^A -globin gene within its 38-kb chromosomal locus is almost completely dependent on the presence of the 3' β -e enhancer, similar to that observed in transfected erythroid cells. Enhancer dependence is achieved by use of chromatin templates reconstituted into nuclei after preincubation with 11-day chick erythroid proteins (lanes 3 and 4) or from repressed templates (lanes 1 and 2) remodeled by replication in the presence of these activator proteins (lanes 5 and 6). β -Globin locus cosmids containing or deleted of the 3' enhancer are replicated identically in these synthetic nuclei. Thus, two separate pathways of forming transcriptionally active chromatin can generate an enhancer response that appears to be stable to transient structural disruption by DNA replication. It is particularly striking that such long-range regulation is re-

Figure 7. Activation of chromatin-repressed β^A -globin gene transcription in synthetic nuclei is enhancer-dependent. Transcription of the adult β -globin gene within the β -globin locus was assessed using cosmids that contained the β - ϵ enhancer residing 2 kb 3' of the β^A -globin gene [Enhancer, +, odd-numbered lanes], and cosmids in which this enhancer was deleted (Enhancer, -, even-numbered lanes), after assembly of these templates into synthetic nuclei. Preincubation in buffer only results in the formation of a repressed chromatin template [RBC^{pre}(-)] that can be activated for transcription by replication in the presence of erythroid activator proteins [RBC^{repl}(+); 20 μ l of 11-day RBC], cf. lanes 1 and 5. This activation is achieved only in the presence of the 3' β - ϵ enhancer, (cf. lanes 5 and 6). Similarly, preincubation with an 11-day RBC extract [RBC^{pre}(+); 20 μ l of 11-day RBC] followed by reconstitution and replication within synthetic nuclei establishes active transcription that is enhancer dependent, (cf. lanes 3 and 4).

capitulated in the synthetic nuclei system with a gene within its actual chromosomal locus and a natural enhancer in its normal position. Smaller β^A -globin gene constructs can also be expressed in an enhancer-dependent manner under the proper assembly conditions (data not shown). We are currently extending this analysis to define the biochemical components and chromatin structural requirements responsible for mediating distal enhancer function in a chromosomal context.

Discussion

We have combined the well-characterized chick β -globin gene family with the ability to form complex nuclear structures by use of *Xenopus* egg extracts, to develop a unique in vitro transcription system for the analysis of mechanisms involved in basic gene expression and tissue-specific regulation. This approach will enable us to broaden the capacity of existing in vitro systems to examine the relationship of a variety of important nuclear processes, such as DNA replication, nuclear scaffold attachment, higher-order chromatin organization, and cell

cycle events to transcription. Our results demonstrate that assembly of the β -globin gene locus into synthetic nuclei, in the presence of staged erythroid extracts, establishes a pattern of β^A -globin transcription under the control of its 3' distal enhancer, which corresponds to that observed during normal erythroid development. Thus, the critical events in tissue-specific and developmentally regulated activation of β^A -globin expression within its chromosomal locus (Evans et al. 1990; Emerson 1993) can be efficiently reproduced in this in vitro system.

Many laboratories have employed *Xenopus* egg extracts as a rich source of histones, nucleosomal assembly factors, and other structural and enzymatic components to study major aspects of nuclear function (Almouzni and Wolffe 1993a). Previous studies demonstrated that *Xenopus* egg extracts can be separated by centrifugation into cytoplasmic and vesicular fractions to independently analyze the processes involved in chromatin assembly, nuclear membrane formation, and membrane-dependent DNA replication (Newport 1987; Sheehan et al. 1988; Vigers and Lohka 1991). The cytoplasmic fraction alone assembles physiologically spaced nucleosomes on DNA but is incapable of double-stranded DNA synthesis because of the lack of membrane vesicles. However, this system has been used to examine the relationship between chromatin activation and single-stranded DNA replication, which is akin to lagging strand DNA synthesis. Experiments with templates of single-stranded 5S genes cloned into M13 vectors provide evidence for a chromatin maturation period following replication when transcription factors may more easily interact with partially assembled H3/H4-bound DNA than with fully mature chromatin (Almouzni et al. 1990a,b). These results, as well as studies with other single-stranded templates or SV40-based DNA replication systems (Wolffe 1991b; Gruss and Sogo 1992; Almouzni and Wolffe 1993b; Kamakaka et al. 1993) have led to models of gene activation in which transcription factor complexes bind in competition with chromatin assembly during the process of DNA replication. As pointed out by Gruss and Sogo (1992), these studies, while illuminating, are the result of in vitro analysis of single-stranded and/or protein-free DNA replication, a situation unlike that in nuclei where chromatin-assembled, double-stranded DNA is replicated during the cell cycle. Although a more complex system, we felt it was worthwhile to model gene activation in reconstituted nuclei because this may more closely resemble in vivo conditions.

We have used fractionated egg extracts to assemble synthetic nuclei in two stages (chromatin formation followed by nuclear membrane encapsulation) to analyze separately the transcriptional regulation of β -globin gene loci in chromatin or synthetic nuclei before and after DNA replication. We find that activation of the β^A -globin gene within its chromosomal locus requires the interaction of developmentally staged erythroid proteins with the promoter and 3' enhancer prior to chromatin assembly. Templates assembled in the absence of ery-

throid proteins are transcriptionally repressed even if these proteins are added later. However, if membrane components are incubated with repressed chromatin to form synthetic nuclei, transcription is activated after a single round of DNA replication in the presence of appropriate concentrations of erythroid factors. This transient disruption of nucleosomal structure creates an opportunity for erythroid proteins to bind β^A -globin DNA and remodel its chromatin configuration to that of an active gene. This effect is completely abolished by the addition of an inhibitor of DNA polymerase α , indicating that activation of repressed genes is dependent on DNA replication and not merely the structural change that occurs as chromatin templates are encapsulated by nuclear membranes.

Interestingly, our experiments show that the passage of a replication fork through active or inactive chromatin does not change the transcriptional capacity of the template. β^A -Globin genes programmed to be active were transcribed efficiently after replication and inactive genes remained repressed. Similar observations were made with 5S RNA genes in *Xenopus* sperm nuclei after replication in egg extracts (Wolffe 1993). Our data suggest that replication can only modulate transcriptional status if specific proteins are available for template binding prior to chromatin re-formation. This has important implications for the establishment of tissue-specific genetic programs, as encoded by particular chromatin structures, and their maintenance during cell division. It predicts that transcriptional programming can remain stable or be modified by regulation of the availability of activators and repressors for a particular gene during the time in S phase when it is replicated. Of course, replication-independent mechanisms of gene activation also exist, particularly for inactive templates that are poised for expression. Several important examples are inducible genes such as rat tyrosine aminotransferase (TAT), yeast PHO5, and *Drosophila hsp70* (Reik et al. 1991; Schmid et al. 1992; Tsukiyama et al. 1994). In the case of hormonal or heat shock-induced genes, chromatin structures can be rendered accessible by either the SWI/SNF protein complex for binding glucocorticoid receptors (Yoshinaga et al. 1992; for review, see Winston and Carlson 1992) or by prior interaction of the GAGA factor for heat shock proteins (Tsukiyama et al. 1994). The relative positioning of factor binding sites and phased nucleosomes may be critical for these and other transactivators (Wolffe 1994). In addition, heterokaryon experiments with both muscle and erythroid cells show tissue-specific gene activation on fusion with nonexpressing fibroblast nuclei in the absence of DNA synthesis (Blau et al. 1983, 1985; Baron and Maniatis 1986, 1991; Blau 1992). However, the majority of genes examined to date are transcriptionally repressed by nucleosome blockage of regulatory regions (Wolffe 1991a; Felsenfeld 1992; Hayes and Wolffe 1992; Adams and Workman 1993), and presumably require DNA replication at some point during cellular differentiation to poise the gene for eventual expression.

Distinct replication-dependent and independent mechanisms

may exist to activate the chromatin structure of genes that are either tissue-specific or subject to rapid induction by hormonal or environmental stimuli. For example, an inducible gene must be maintained in a chromatin structure that allows it to be transiently repressed but able to respond immediately to external signals that are not predetermined by the cell. The requirement for DNA replication to activate an inducible gene is far too costly in time, and it is more expedient to generate a chromatin structure that is repressed but poised for transcription. The generation of this poised structure may have involved DNA replication at some point but the final stage of gene activation is replication independent. By contrast, tissue-specific gene expression in particular cells and at precise times during development is predetermined during cellular differentiation and is not subject to rapid, unprogrammed activation and inactivation. Although the chromatin structure of tissue-specific genes may also be poised for transcription, it is more reasonable to assume that replication-dependent activation is involved with genes whose timing of expression is predictable within a specific tissue.

The role of distal enhancer elements in stabilizing transcription complex binding during the interplay of replication and chromatin assembly has been a speculative one (Gross and Garrard 1987; Felsenfeld 1992). Our results show a vital role for the 3' distal enhancer in the control of β^A -globin gene transcription within synthetic nuclei. Enhancer function can be generated on chromatin templates that are either preactivated or activated by structural remodeling after replication. Deletion of the enhancer abolishes transcription from both templates. Interestingly, enhancer activity is completely stable to DNA replication, indicating that promoter-enhancer communication is rapidly re-established after transient chromatin disruption. Further experimentation and biochemical analyses are necessary to define the chromatin structural features that mediate long-range interactions and to determine whether enhancer activity must be maintained throughout transcription or is needed transiently to activate initiation, as has been described previously for SV40 (Wang and Calame 1986). In addition, gene switching within the β -globin locus by differential interaction between the adult β and embryonic ϵ promoters and the shared β - ϵ enhancer can now be actively explored in this system.

Questions of domain organization, gene commitment, and memory during development and differentiation have remained refractory to in vitro analysis (Brown 1984; Weintraub 1985; Gross and Garrard 1987). We hope that synthetic nuclei will provide a valuable system to dissect these and other nuclear processes, both structurally and biochemically, and determine how they influence the transcriptional regulation of any cloned gene or chromosomal region.

Materials and methods

Plasmid constructions

Construction and characterization of the chick β -globin locus

clone sCos5 β A1, which encompasses the entire β -type globin locus from map units -7.2 to $+28$ [see Reitman and Felsenfeld 1990], has been described previously [Barton et al. 1990]. The adult β -globin gene within the cluster was mutated by insertion of a 17-bp polylinker sequence at $+46$ relative to the start-site of β -globin transcription [Emerson et al. 1989; Barton et al. 1990] to distinguish in vitro-generated transcripts from the endogenous β -globin RNA present in the RBC protein extracts employed. Deletion of the β - ϵ shared enhancer from sCos5 β A1 was achieved by protection of the *HhaI* restriction endonuclease sites at $+1620$ and $+2098$ by RecA protein-mediated triple helix formation with oligonucleotides (30-mers) encompassing these sites, methylation of the remaining *HhaI* sites within the cosmid by *HhaI* methylase, and then cleavage with *HhaI* at the unmethylated $+1620$ and $+2098$ sites after removal of the RecA complexes under conditions described in the RARE method of mutagenesis [Ferrin and Camerini-Otero 1991].

Xenopus egg extracts

Unactivated *Xenopus* egg extracts that are competent to assemble cloned DNA into chromatin were prepared exactly as described by Barton et al. (1993). The clarified egg cytoplasm was fractionated by centrifugation into soluble cytoplasmic and vesicular membrane fractions and collected by side puncture of the centrifugation tube according to the procedure of Wilson and Newport (1988). The unfractionated, clarified egg cytoplasm was used to assess nuclear reconstitution around the chick β -globin locus DNA as described [Newport 1987]. One microgram of DNA was incubated in 100 μ l of the unfractionated cytoplasmic extract at 22°C. Aliquots of 2 μ l were removed at specific times and mixed with 2 μ l of the DNA-specific fluorescent stain bisbenzimidazole (1 μ g/ml; Sigma) described in Newport (1987). DNA and nuclear structures were analyzed by fluorescent and phase-contrast microscopy at a magnification of 400 $\times$.

In vitro transcription

Prior to nucleosome assembly or synthetic nuclei formation, chick β -globin cosmids (0.5 μ g) were preincubated (RBC^{pre} incubation) for 20 min at room temperature with RBC extract dialysis buffer or varying amounts of 11-day RBC protein extracts (35 mg/ml protein concentration) prepared as described previously [Emerson et al. 1989]. A *Xenopus* egg cytoplasmic soluble fraction in an amount previously determined to fully repress transcription and fully supercoil relaxed chick β -globin DNA templates (generally 12.5 μ g/ml final protein concentration), plus a final concentration of 3.5 mg/ml purified *Xenopus* egg glycogen [Hartl et al. 1994], were added to assemble DNA templates into chromatin for 1 hr at 22°C. Transcription of these nucleosome-assembled templates in the absence of membranes occurred as described [Barton et al. 1993] on the addition of a RNA polymerase II-containing RBC extract [Emerson et al. 1989].

Reconstitution of chromatin-assembled templates into synthetic nuclei was achieved by addition of a *Xenopus* egg membrane vesicular fraction (60 μ g of protein) plus extract buffer (XBII buffer, Barton et al. 1993) prior to the transcription extract. Additional RBC protein extract (35 mg/ml) in varying amounts (see figure legends) and RBC extract dialysis buffer (see above) were added to a total volume of 60 μ l. DNA replication and chromatin remodeling within synthetic nuclei occurred over a 2-hr period at 22°C. (RBC^{rep} incubation period). Transcription was initiated by addition of transcription extracts (generally 10 μ l of a 35 mg/ml protein concentration extract) and an NTP/

salts/energy-generating mix to give final concentrations of 0.7 mM CTP, UTP, GTP, 1.0 mM ATP, 5.8 mM MgCl₂, 50 mM KCl, 5 mM creatine phosphate, 10 U/ml of creatine kinase, and 12.6 mM HEPES (pH 7.9). The final volume of the synthetic nuclei transcription reaction under these conditions was between 87–93 μ l. After a 60-min incubation at 30°C, RNA products were purified and analyzed by S1 nuclease digestion and gel electrophoresis [Emerson et al. 1989].

DNA replication

Replication of DNA occurs after the addition of ~ 60 μ g protein of the membrane vesicle fraction to 0.5 μ g of chromatin-assembled cosmids and the formation of an intact nuclear membrane. Replication was assayed by continuous labeling of newly synthesized DNA with 1 μ Ci of [α -³²P]dATP (3000 Ci/mmol, Amersham) added with membranes (time = 0) to a total volume of 60 μ l under the exact conditions described for in vitro transcription reactions. Aliquots were removed from the reaction at specific times and were either spotted directly onto DE81 filters (Whatman), which were phosphate buffer washed and counted as described [Blow and Laskey 1986], or made protein-free prior to ethanol precipitation and electrophoretic analysis on a 0.8% TBE-agarose gel.

To monitor uniformity of replication, a Southern blot analysis was performed on restriction enzyme digests of 0.5 μ g of sCos5 β A1 DNA separated on an 0.8% TBE-agarose gel. The gel was blotted onto GeneScreen Plus membrane (New England Nuclear) by standard procedures and hybridized with an entire DNA replication reaction (0.5 μ g of cosmid DNA in a final reaction volume of 60 μ l) labeled with 10 μ Ci of [α -³²P]dATP, as described above. Hybridization and washing conditions were as described [Church and Gilbert 1984].

Chromatin structural analysis

Parallel incubations of nucleosome-assembled chick β -globin locus DNA (0.5 μ g) in the absence of membranes (plus 50 μ g/ml aphidicolin) or reconstituted into nuclei were performed in the presence or absence of 1 μ Ci [α -³²P]dATP, exactly as described for transcription assays, and then digested with staphylococcal nuclease. Staphylococcal nuclease digests of nucleosome-assembled templates and reconstituted synthetic nuclei were conducted as described by Barton et al. (1993). One-tenth volume aliquots were removed at specific times of digestion and the reactions stopped with 0.02 M EDTA/0.002 M EGTA. Similarly, limit digestion to mononucleosomes was achieved by a 2-hr incubation period under these conditions. Proteins were extracted and the DNA separated on a 1.5% TBE-agarose gel. Unlabeled DNA protected by nucleosome assembly from staphylococcal nuclease digestion was analyzed by Southern blot analysis and probed with random-primed ³²P-labeled pUC18ABC/ Δ 1 DNA (containing a 4.2-kb insert of the entire chick β^A -globin gene; Emerson et al. 1989) as described in Barton et al. (1993). Newly synthesized DNA labeled by ³²P incorporation during replication was subjected to electrophoresis on the same 1.8% TBE-agarose gel and then separately blotted and exposed to X-ray film.

MspI cleavage sensitivity

MspI digestion analyses were performed after reconstitution of 0.5 μ g chick β -globin cosmid DNA into synthetic nuclei under the conditions described above for in vitro transcription reactions. Following the 1 hr chromatin assembly period and the 2-hr replication and coupled chromatin assembly of nuclei-reconstituted DNA, *MspI* accessibility of the adult β -globin gene promoter was assayed essentially as described by Emerson and

Felsenfeld (1984) with minor changes. MgCl_2 was added to a final concentration of 10 mM and 27 units of *MspI* added to digest DNA at 37°C for 1 hr. Purification and separation of digested DNA on a 1.8% TBE-agarose gel followed by Southern blot analysis was performed as described by Barton et al. (1993). A gel-isolated *MspI* fragment from the -109 to -224 region of the chick β -adult promoter was labeled by reverse transcriptase fill-in in the presence of [α - ^{32}P]dCTP (3000 Ci/mMole, Amersham) and used to probe the Southern blot of the *MspI*-digested DNA.

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