The erythroid protein cGATA-1 functions with a stage-specific factor to activate transcription of chromatin-assembled β -globin genes

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The chick β -globin gene is regulated developmentally within erythroid cells by the interaction of multiple proteins with the promoter and the 3' enhancer. These interactions are correlated with changes in chromatin structure, which are characteristic of the actively expressed gene. Using in vitro chromatin assembly and transcription with staged erythroid extracts, we have determined the critical proteins required to activate expression of nucleosome-reconstituted β -globin genes. These genes contain a specialized TATA box at -30 (GATA) through which the erythroid-restricted protein cGATA-1 and TFIID both function to regulate different steps in β -globin expression. We find that TBP (TATA-binding protein) alone can activate transcription of β -globin chromatin templates from promoters mutated to a canonical TATA box but is ineffective on those containing the normal -30 GATA site. The occupancy of this site by cGATA-1 also fails to generate efficient expression of β -globin chromatin unless combined with a stage-specific protein, NF-E4, that binds to an adjacent site. However, NF-E4 does not function with TBP to derepress nucleosome-assembled β -globin genes. We propose that the developmental regulation of β -globin expression is achieved, in part, by the requirement of an erythroid protein and a stage-specific factor, rather than TBP, to activate chromatin through a specialized TATA box.

[**Key Words:** Chromatin activation; erythroid transcription factor, GATA-1, NF-E4; β -globin gene transcription]

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The chick β -globin gene family (5'- ρ - β^H - β - ϵ -3') is transcriptionally regulated at defined developmental stages in erythroid cells. This is accompanied by specific changes in the chromatin structure of individual genes (Stalder et al. 1980; McGhee et al. 1981; Reitman and Felsenfeld 1990). The two embryonic β -globin genes, ρ and ϵ , are expressed in primitive red blood cells (RBCs) at approximately day 5 of chick embryonic development, whereas the adult β -globin genes, β^H and β , are active only in definitive RBCs beginning at about day 9 (Bruns and Ingram 1973; Brown and Ingram 1974; Chapman and Tobin 1979). In erythroid cells, the β -globin gene locus is maintained in a DNase I-sensitive chromatin structure that is characterized by multiple nuclease hypersensitive sites distributed throughout the locus. Many of these sites appear stage specifically in the promoter and enhancer regions of individual globin genes at the time of their expression and represent localized disruptions in the normal nucleosomal array. For example, the adult β -globin 3' enhancer is in a hypersensitive chromatin

structure throughout erythroid development, whereas the promoter region is blocked by a nucleosome until the onset of transcription in definitive RBCs (Stalder et al. 1980; McGhee et al. 1981; Reitman and Felsenfeld 1990). The β - ϵ enhancer is situated between the β - and ϵ -globin genes and must remain presumably accessible throughout erythroid development to regulate each promoter at different stages (Choi and Engel 1988; Nickol and Felsenfeld 1988). Chromatin reconstitution experiments have shown that these tissue- and stage-specific hypersensitive regions can be generated on cloned β -globin genes by the interaction of nuclear proteins from definitive RBCs on the promoter and 3' enhancer. In contrast, proteins from primitive RBCs or nonerythroid cells fail to confer nuclease hypersensitivity (Emerson and Felsenfeld 1984). Thus, the interaction of specific proteins with critical transcriptional control regions is sufficient to remodel nucleosomal structures to resemble those associated with active genes.

The β -globin promoter and enhancer regions bind multiple proteins, consisting of ubiquitous, tissue-specific, and developmental stage-specific factors, the composition of which changes during erythroid development

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(Emerson et al. 1985, 1987, 1989; Plumb et al. 1986; Evans et al. 1988; Lewis et al. 1988). In vitro transcription experiments have shown that extracts prepared at different times in RBC maturation express cloned β -globin genes in a manner similar to that observed with the endogenous gene in vivo (Emerson et al. 1989). Transcriptional regulation occurs through a combination of activators that are most concentrated in RBCs of the correct developmental stage. Of particular interest are two proteins that interact near the initiation site of transcription in the β -globin promoter, GATA-1 and NF-E4. GATA-1 is an erythroid-restricted protein that is required for β - ϵ enhancer function (Emerson et al. 1987; Evans et al. 1988; Reitman and Felsenfeld 1988) and has a critical role in the regulation of many erythroid genes (Evans et al. 1988; Plumb et al. 1989), as well as in the differentiation of the RBC lineage (Pevny et al. 1991). NF-E4 is a stage-specific factor found in definitive RBCs that has been implicated in mediating the switch from ϵ - to β -globin expression by increasing the affinity of the β -globin promoter for the β - ϵ enhancer and, thereby, decreasing its activation of the ϵ -promoter (Gallarda et al. 1989; Foley and Engel 1992). NF-E4 has also been shown to function as a transcriptional activator of β -globin genes in vitro (Emerson et al. 1989).

Recent studies have indicated that most members of the β -globin gene family contain a noncanonical TATA box at -30 , the sequence of which is GATA. This specialized TATA box has important functional consequences because the GATA-1 protein can regulate β -globin expression through the -30 initiation site as well as through the $3'$ enhancer upon transfection into primary chick erythroid cells. Both GATA-1 and transcription factor IID (TFIID) are required for efficient β -globin transcription by interacting with the -30 GATA box at different times and performing distinct functions. Initially, GATA-1 mediates promoter-enhancer interaction by binding to sites within each element. Once this interaction is stabilized, TFIID forms a complex with adaptor proteins and displaces GATA-1 from the -30 GATA box to initiate transcription. When bound to the -30 site, GATA-1 does not affect promoter activity directly, unlike NF-E4, but confers erythroid specificity to the initiation complex by recruiting the distal enhancer (Fong and Emerson 1992). These and other aspects of β -globin regulation are not observable on naked DNA, suggesting that specific nuclear structures may be required. To better understand the nature of these structures and how erythroid proteins function within them, we analyzed the transcriptional regulation of chromatin-assembled β -globin genes.

Here we show that β -globin genes reconstituted into chromatin are repressed transcriptionally, but can be tissue-specifically activated by preincubation with erythroid extracts. This activation is erythroid stage specific as the extracts must be prepared from early definitive RBCs that express β -globin RNA efficiently. Proteins from primitive or mature RBCs or nonerythroid tissues fail to activate nucleosome-assembled β -globin genes, presumably because factors from these cells cannot ren-

der the promoter accessible to RNA polymerase and other basal transcription factors. Analysis of promoter deletions indicates that only a minimal sequence, containing the binding sites for GATA-1 and NF-E4, is required to activate expression of β -globin chromatin templates by RNA polymerase II. Preincubation of these two purified proteins with β -globin genes before nucleosome assembly is sufficient to alleviate chromatin-mediated transcriptional repression. In contrast, TFIID cannot derepress β -globin genes by replacing GATA-1 at the -30 site, unless this sequence is mutated to a canonical TATA box. Thus, tissue- and developmental stage-specific expression of chromatin-assembled β -globin genes is achieved by the combined action of an erythroid-restricted protein that binds to a specialized TATA box, and a factor present only in definitive RBCs.

Results

Chromatin-assembled β -globin genes are transcriptionally derepressed by erythroid proteins

To examine the role of chromatin structure in the erythroid-specific activation of β -globin expression, we assembled these genes into nucleosomes using extracts from *Xenopus* eggs. The transcriptional activity of the reconstituted templates was then monitored by incubation with 11-day RBC extracts that express cloned β -globin genes in vitro (Emerson et al. 1989). *Xenopus* egg extracts have been shown to assemble histone octamers efficiently onto DNA with the appropriate spacing (for review, see Rhodes and Laskey 1989). The mild reaction conditions used in this procedure are especially important because complexes between erythroid proteins and β -globin genes must be preformed and stable during chromatin assembly, as these proteins cannot bind to nucleosomal DNA. This requirement precludes the use of harsher methods of assembly involving high salt dialysis or chemical agents that might disrupt the prebound protein-DNA complexes. As shown in Figure 1B, incubation with increasing amounts of the *Xenopus* assembly extract results in the progressive repression of β -globin transcription (lanes 2–5) compared with that of unassembled DNA (lane 1). This is attributable to the assembly of the DNA templates into chromatin and not to a general inhibition of the transcription machinery.

To activate repressed β -globin genes transcriptionally, the chromatin structure must be altered to allow efficient initiation by RNA polymerase II. This structural change presumably creates an accessible, or nucleosome-free, region in the promoter to allow the binding of RNA polymerase II and general initiation factors. Here, we use the terms derepression and activation synonymously and define them as the ability of proteins to modify nucleosomal structures to allow high-level transcription from assembled genes relative to unmodified chromatin not to free DNA. To test this hypothesis, we examined the ability of erythroid nuclear proteins to activate transcription by modifying the structure of chromatin assembled on β -globin DNA according to the protocol in

Figure 1. Repression of transcription by chromatin assembly is relieved by preincubation of β -globin genes with 11-day RBC proteins. (A) Schematic diagram of the nucleosome assembly and in vitro transcriptional analysis of chick β -globin genes. DNA templates were preincubated for 20 min at room temperature with 11-day chick RBC protein extracts, purified proteins, or buffer. After this incubation, a nucleosome assembly extract from *Xenopus* eggs was added to the DNA and chromatin allowed to form for 60 min at 22°C. Transcription was initiated by the addition of an RNA polymerase II-dependent RBC extract from 11-day chick embryos and an NTP/salt/energy generation mix. The amount of β -globin RNA synthesized in these reactions was determined by S1 nuclease analysis (see Materials and methods). (B) In vitro transcription of chromatin-assembled β -globin genes: repression of transcription by nucleosome assembly and formation of transcriptionally active β -globin templates with RBC nuclear proteins. DNA templates were assembled with increasing amounts of *Xenopus* egg extract (X.1. egg; 50 mg/ml) from 0–25 μ l (lanes 1–5, respectively). The concentration of *Xenopus* extract required to repress transcription completely, 25 μ l (lane 5), was used to assemble β -globin DNA into chromatin (lanes 6–11). These reactions were preincubated with increasing concentrations of 11-day RBC nuclear proteins (RBC; 37 mg/ml), from 0–20 μ l (lanes 6–10, respectively). After preincubation, the β -globin chromatin templates were transcribed with an erythroid-specific transcription extract under the conditions described (see Materials and methods). Sensitivity to low levels of α -amanitin was assayed under the exact conditions as shown in lane 10 with the addition of 100 ng/ml of α -amanitin (lane 11). Transcription reactions were quantitated by scanning the autoradiograms after S1 nuclease analysis. The values obtained are shown below each lane in comparison with the RNA transcripts of lane 5 for repression and lane 6 for activation. In vitro-synthesized β -globin transcripts and endogenous β -globin RNA present in the 11-day RBC extracts are designated by arrows. (C) In vitro transcription of chromatin-assembled β -globin genes is absolutely dependent on the addition of an RNA polymerase II transcription extract after nucleosome assembly. A control experiment was performed, essentially as described above, in which *Xenopus* egg extract was added to assemble chromatin (0–20 μ l, lanes 1–4, respectively, and 20 μ l, lanes 5–8) on β -globin templates preincubated with buffer only (lanes 1–4) or increasing concentrations of 11-day RBC nuclear proteins, 0, 10, 15, and 20 μ l (lanes 5–8, respectively). After assembly, the β -globin chromatin templates were transcribed with an RNA polymerase II-dependent nuclear transcription extract as described above (lanes 1–4), or incubated in nuclear extract buffer only in place of the transcription extract (lanes 5–8).

Figure 1A. Incubation of β -globin genes with increasing amounts of 11-day RBC nuclear proteins before nucleosome assembly results in efficient derepression of chromatin templates (Fig. 1B). These RBC proteins consist of multiple factors that interact with both the β -globin promoter and the 3' enhancer (Emerson et al. 1987,1989) and prevent the formation of nucleosomes on these critical control regions (Emerson and Felsenfeld 1984; Emerson et al. 1987). The generation of "open" complexes results in increased transcription proportional to the

amount of RBC nuclear proteins added until saturation of the DNA templates occurs (Fig. 1B, lanes 6–10). The level of expression of the chromatin-assembled templates is equal, when fully saturated, to that obtained with unassembled DNA (Fig. 1B; cf. lane 1 with lanes 9–10).

In this system, RNA synthesis is completely dependent on the addition of erythroid transcription extracts after chromatin assembly (Fig. 1C, lanes 1–4) as the *Xenopus* egg extracts are not competent to express β -globin

genes (Fig. 1C, lanes 5–8). As shown in Figure 1B, β -globin transcription is abolished by the presence of low levels of α -amanitin (lane 11), indicating that RNA polymerase II, in the erythroid extracts, is required for gene expression. Apparently, transcription does not occur during nucleosome assembly as α -amanitin is added after incubation with the *Xenopus* extract, before the addition of the RNA polymerase II-containing erythroid extract. Any RNA produced in vitro from unassembled DNA templates during this time would be detected as an RNA signal (Fig. 1B, lane 11). Similarly, no RNA is synthesized during the preincubation step with RBC nuclear proteins (Fig. 1B, lane 11, and C, lanes 5–8).

These results indicate that the activation of chromatin-assembled β -globin genes requires the binding of erythroid proteins before nucleosome formation. Once these proteins are bound to the promoter, nucleosomes are excluded from this region and the modified chromatin structure is accessible to the formation of an RNA polymerase II-dependent initiation complex. Interestingly, neither erythroid proteins nor general transcription factors added after nucleosome assembly are able to activate β -globin expression (Fig. 1B, lane 5). This suggests that these proteins cannot bind to nucleosomes or displace them. Once chromatin is formed on the β -globin gene, transcription is repressed as a result of the inaccessibility of activator protein-binding sites.

Repression of gene activity is correlated with a change in DNA structure in the presence of the *Xenopus* extract (Fig. 2A). As increasing amounts of the assembly extract are added, the DNA topology of β -globin genes shifts from the supercoiled form of free DNA (lane 1) to progressively relaxed forms (lanes 2–5) and back to complete supercoiling as nucleosomes are fully loaded onto the DNA (lanes 6–8). This pattern is characteristic of topological perturbations in DNA structure attributable to coiling of DNA around nucleosomes during chromatin assembly. Preincubation of β -globin genes with RBC nuclear proteins does not inhibit the ability of *Xenopus* extracts to form supercoiled DNA (cf. lanes 10 and 11 with lanes 12 and 13). Further analysis of the nucleosomal structure of the reconstituted templates is provided by staphylococcal nuclease digestion (Fig. 2B). After incubation of β -globin genes in the *Xenopus* extract and assembly into chromatin, increasing amounts of staphylococcal nuclease were added to reveal a regular pattern of the nucleosomal array (lanes 1–5). The spacing of nuclease protection of ~ 180 nucleotides and its ordered arrangement indicate that chromatin structures similar to those found in vivo are formed with this *Xenopus* system. To assess whether the presence of RBC nuclear extracts interferes with chromatin formation, a limit staphylococcal digest was performed on DNA assembled with *Xenopus* extracts alone or in the presence of erythroid proteins. Comparison of the relative amounts of mononucleosomes formed when assembled in the presence of either RBC nuclear extracts (Fig. 2C, lane 2) or purified DNA-binding proteins, mGATA-1 and NF-E4 (Fig. 2C, lane 3), indicates that these conditions did not disrupt, but slightly enhanced, the efficiency of nucleo-

some formation relative to that obtained with the *Xenopus* extract alone (Fig. 2C, lane 1). Analysis of the migrations of these same mononucleosome populations through a sucrose density gradient (Fig. 2D) revealed no gross effects on nucleosome structure or number when assembled onto β -globin DNA in the presence of RBC proteins. These analyses and the supercoiling studies demonstrate that the mechanism by which RBC proteins transcriptionally derepress chromatin-assembled β -globin genes is not by bulk nucleosomal loss.

Transcriptional activation of β -globin chromatin is tissue and developmental stage specific

The nuclear proteins that activate β -globin transcription efficiently in chromatin templates were extracted from definitive erythroid cells at a stage in which the endogenous β -globin gene is maximally expressed (11-day chick embryos). To examine whether activation is specific to proteins isolated at this time in development, we analyzed nuclear extracts from different stages of erythroid maturation and from nonerythroid cells for their ability to render nucleosome-assembled β -globin genes transcriptionally competent. Protein extracts from primitive 5-day RBCs, which express the endogenous embryonic globin genes ρ and ϵ , adult RBCs, which are transcriptionally quiescent, and chick brain, which is inactive for globin expression, were used. β -Globin DNA was preincubated with each of these nuclear extracts, assembled into nucleosomes, and transcribed as described previously (see Fig. 1). As shown in Figure 3A, proteins isolated from tissue sources that do not express the endogenous β -globin gene fail to form chromatin templates that are accurately and efficiently transcribed in vitro. For example, β -globin chromatin templates assembled in the presence of brain extracts produce very low levels of specific transcripts (lanes 9–11), whereas proteins from staged erythrocytes, 5-day and adult, generate β -globin chromatin templates that are more efficiently transcribed than from heterogeneous start sites (lanes 3–8). The difference in overall transcriptional efficiency between chromatin assembled with proteins from non- β -globin-expressing erythrocytes and brain may reflect the fact that the 5-day and adult RBC extracts contain a subset of the activator proteins present in 11-day RBCs. These activators increase the transcriptional potential of β -globin chromatin by partially “opening” the promoter region. However, the heterogeneity of start sites suggests that proper initiation may require components that are present only at the appropriate stage of RBC development. Our results indicate that proteins present in 11-day RBC extracts are sufficient to form transcriptionally active β -globin chromatin and to determine the proper site of initiation (lane 2). Thus, tissue- and stage-specific expression of nucleosome-assembled β -globin genes is conferred by a combination of proteins that are developmentally regulated.

Further evidence of specific changes in the promoter region of β -globin chromatin templates that accompany

Figure 2. Preincubation of the β -globin template with 11-day RBC proteins does not disrupt nucleosome assembly. (A) Supercoiling analysis. β -Globin DNA was assembled as described with increasing concentrations of *Xenopus* egg extract. (Lanes 1–8) The DNA topology after nucleosomal assembly with 0, 2.5, 5, 7.5, 10, 15, 20, and 25 μ l of egg extract for 1 hr at 22°C. (Lanes 9–14) The effect of RBC preincubation on DNA topology. Nucleosome assembly by *Xenopus* egg extract (lanes 9, 14, 0 μ l; lanes 10, 12, 2.5 μ l; lanes 11, 13, 25 μ l) was preceded by incubation in buffer only (lanes 9–11) or 15 μ l of an 11-day RBC extract (lanes 12–14). The migration of supercoiled (s.c.) and relaxed (r.f.) forms of DNA are designated by the appropriate arrows. (B) Staphylococcal nuclease analysis. (Lanes 1–5) β -Globin DNA was preincubated as described above with buffer only. After the chromatin assembly of β -globin DNA as described with 25 μ l of *Xenopus* egg extract, the DNA was digested with staphylococcal nuclease for 0, 2.5, 5, 15, and 30 min (lanes 1–5, respectively). The DNA protected from nuclease digestion by the binding of nucleosomal core proteins was purified and analyzed as detailed in Materials and methods. Nucleosome spacing was estimated by comparison with a 123-bp DNA ladder (lane M). (C) Staphylococcal nuclease limit digests in the presence of RBC proteins. (Lane 1) β -Globin DNA was assembled into chromatin and digested with staphylococcal nuclease for 2 hr, as described above, after preincubation with buffer only; (lane 2) 30 μ l of an 11-day RBC extract/ μ g of DNA; or (lane 3) 10 footprinting units (fpu) each of mGATA-1 and NF-E4 proteins/ μ g DNA. Analysis of protected DNA fragments was performed as described above using 200 ng of the starting DNA material. (D) Sucrose gradient analysis of mononucleosome populations. The mononucleosome protein–DNA complexes that were the result of the preincubation/assembly/staphylococcal nuclease digestion conditions presented above (Fig. 2C; lanes 1, 2) were purified through parallel 5–29% sucrose gradients as described in Materials and methods. A 20- μ l portion of every other fraction collected after centrifugation was analyzed on a 1% agarose gel to determine the relative migration of the mononucleosomes formed on β -globin DNA preincubated in the presence (lower gel) or absence (upper gel) of 11-day RBC proteins.

transcriptional activation is provided by *MspI* cleavage sensitivity of the β -globin promoter (Fig. 3B). Earlier studies demonstrated that the 5'-flanking region of the chick β -globin gene becomes nuclease hypersensitive in definitive RBC (9-day to adult) as a result of the loss of a nucleosome over the promoter region concomitant with the onset of expression (McGhee et al. 1981). This developmentally regulated chromatin structural change can be generated in vitro by the interaction of nuclear proteins from definitive RBC extracts with cloned β -globin genes before nucleosome assembly (Emerson and Felsenfeld 1984). In both types of analyses, digestion with *MspI* endonuclease results in the specific release of a 115-bp fragment from the β -globin promoter when this region is

nucleosome-free in either RBC nuclei or chromatin-reconstituted templates. The results presented in Figure 3B indicate that a nucleosome-free region of the β -globin gene promoter is formed under chromatin assembly conditions that generate DNA templates having maximal transcription efficiency. This occurs only when β -globin genes are preincubated with nuclear extracts or DNA-binding proteins from 11-day RBC (Fig. 3B, lanes 3–7). The promoter region does not become accessible in the presence of nuclear proteins from 5-day RBC or chick brain (lanes 8–13). This hallmark of an open promoter conformation suggests that transcriptional activation of the chick β -globin gene is a result of nucleosome exclusion from the 5'-flanking region attributable to the in-

Figure 3. Transcriptional activation of chromatin-assembled β -globin genes is tissue and developmental stage specific and is accompanied by the appearance of a hypersensitive region in the promoter. (A) In vitro transcription of chromatin-assembled β -globin genes preincubated with tissue- and stage-specific protein extracts. β -Globin DNA was incubated before chromatin assembly with nuclear protein extracts from different stages of RBC development and from a nonerythroid tissue. The concentration of extract added to each reaction is the following: (Lane 1) buffer alone; (lane 2) 15 μ l of an 11-day RBC nuclear extract; (lanes 3–5) 5, 15, 25 μ l of extracts from 5-day RBCs; (lanes 6–8) adult RBCs; and (lanes 9–11) chick brain cells. All nuclear extracts had protein concentrations of 35–40 mg/ml. After this preincubation step, chromatin assembly and in vitro transcription were performed as described. (B) *MspI* cleavage sensitivity of the β -globin promoter in chromatin reconstituted templates. β -Globin genes were assembled as described above for in vitro transcription analyses. The chromatin templates were then cleaved with *MspI* and processed as described in Materials and methods. The specific release of a 115-bp promoter fragment (arrow) was assayed by probing with a fragment of the chick β -globin promoter (Chick Globin Msp 115; lanes 1–13) or, as a negative control, re-probed with fragments of pBR322 (pBR Msp 110/123; lanes 14–20). (Lane 1) The *MspI* digest of nucleosome-free β -globin DNA. (Lanes 2–13) The *MspI* digests of chromatin-assembled DNA that was reconstituted in *Xenopus* extracts after preincubation in buffer only (lane 2); 5, 10, 15 μ l of an 11-day RBC nuclear extract (lanes 3–5); 2 and 4 μ l of a DNA–cellulose fraction (DC fxn; 0.56 mg/ml) containing the promoter-binding proteins present in an 11-day RBC nuclear extract (lane 6, 7); 5-day RBC extract (lanes 8–10); and chick brain extract (lanes 11–13). The blot of lanes 1–7 was stripped and re-probed with pBR322 fragments as a control for the nonspecific release of *Msp* digestion products in the 115-bp size range (lanes 14–20).

teraction of specific activator proteins present at the appropriate stage of erythroid development.

A minimal promoter sequence is required to activate expression of chromatin-assembled β -globin genes

To determine which β -globin promoter-binding proteins are essential for transcriptional activation of chromatin-assembled genes, we analyzed templates containing deletions of specific factor-binding sites. The complete pro-

motor to –407 interacts with at least eight proteins in β -globin-expressing 11-day RBC extracts, as summarized in Figure 4A. These factors are mainly transcriptional activators with different individual tissue distributions. For example, the BGP1 and Sp1 proteins are present in both erythroid and chick brain extracts and appear not to affect transcription directly as their binding sites (–195 to –185) can be deleted without decreasing β -globin expression (Emerson et al. 1989; Jackson et al. 1989). In contrast, the CACC, β -AP2, β -CTF, and NF-E4 proteins

Figure 4. A minimal promoter sequence is sufficient to generate transcriptionally active β -globin chromatin. (A) Schematic diagram of the chick β -globin promoter region and its associated proteins. The complete promoter interacts with at least eight proteins in 11-day RBC nuclear extracts. Basal level transcription of chromatin-assembled genes is conferred by the binding of the erythroid-restricted protein cGATA-1 to the -30 "specialized" TATA box. This low activity is increased significantly by the interaction of several developmental stage-specific factors, NF-E4 (-50), β -CTF (-70), and β -AP2 (-90). The CACC factor (-135) also functions as a transcriptional activator but deletion of the BGP1 and Sp1 protein-binding sites (-185 and -195) has little effect on β -globin expression from nucleosome-assembled templates. (B) Transcriptional levels of chromatin-assembled β -globin genes containing sequential promoter deletions or point mutations were compared with genes having the full promoter. All DNA templates were preincubated with $20 \mu\text{l}$ of 11-day RBC nuclear proteins (37 mg/ml), assembled into chromatin, and transcribed in vitro with erythroid extracts as described. β -Globin genes with the full promoter (-407) were transcribed as follows: (lane 1) free DNA; (lane 2) nucleosome assembled; (lane 3) nucleosome assembled with RBC nuclear proteins; and (lane 4) plus 100 ng/ml α -amanitin. β -Globin templates with sequential deletions or point mutations in the promoter were preincubated with RBC nuclear proteins, nucleosome assembled, and analyzed by in vitro transcription in the following order: (Lane 5) -120 ; (lane 6) -58 ; (lane 7) plus 100 ng/ml α -amanitin; and (lane 8) -39 ; (lane 9) plus 100 ng/ml of α -amanitin; (lane 10) GG; (lane 11) TATA; and (lane 12) $-NF-E4$.

that interact between -135 and -50 are essential transcriptional activators both in vitro and in transfected erythroid cells. Interestingly, these factors are regulated in a stage-specific manner as their concentrations are highest in definitive RBCs and evidence exists that they are tissue-specific members of different multi-gene families (Emerson et al. 1989; Gallarda et al. 1989; Jackson et al. 1989; Foley and Engel 1992). This combination of proteins activates transcription through the initiation complex formed at the noncanonical TATA box (GATA) at -30 . This sequence interacts with the erythroid-restricted protein, cGATA-1, and with the TATA-binding protein, TBP, in a sequential manner during β -globin transcription. cGATA-1 does not function as a direct transcriptional activator at this site, but is required for enhancer-dependent β -globin expression (Fong and Emerson 1992).

β -Globin genes containing sequential deletions and point mutations in the promoter region were used to identify the protein-binding sites that are critical for the expression of nucleosome-repressed templates. These genes were preincubated with 11-day RBC nuclear proteins, assembled into chromatin, and analyzed by in vitro transcription in erythroid extracts. As shown in

Figure 4B, β -globin templates containing the full promoter to -407 are completely repressed when assembled into chromatin (lane 2), but can be activated by preincubation with 11-day RBC nuclear proteins (lane 3) and expressed with a slightly greater efficiency than free DNA (lane 1) in an RNA polymerase II-dependent manner (lane 4). β -Globin genes with promoters deleted to -120 (lane 5) show a modest decrease in transcription compared with templates containing the full promoter (lane 3) primarily as a result of the loss of the CACC activator protein-binding site. The BGP1 and Sp1 proteins that bind between -180 and -200 have very little effect on β -globin transcription in vitro either as free DNA (Emerson et al. 1989) or when assembled into chromatin (data not shown). In contrast, a deletion to -58 reduces expression severalfold (lane 6), demonstrating the importance of the binding sites for the stage-specific activator proteins β -AP2 and β -CTF. A further deletion to -39 , which eliminates NF-E4 binding, decreases transcription dramatically to a negligible level (lane 8) that is still correctly initiated. The NF-E4 protein is critical in activating the -30 cGATA-1 complex as promoters containing a point mutation in the NF-E4-binding site ($-NF-E4$; lane 12) are lower in activity than those de-

leted to -58 (lane 6), even when the other stage-specific activators remain bound.

These results indicate that the interaction of cGATA-1 alone at the -30 GATA box is sufficient for correctly initiated, low-level transcription from chromatin templates and that the developmentally regulated factors NF-E4, β -AP2, and β -CTF significantly increase the activity of this basal initiation complex. The importance of the -30 GATA site is demonstrated by the observation that a mutation that prevents the binding of cGATA-1 within the full promoter (GG; lane 10) completely abolishes transcription, unlike the other promoter mutations that only decrease β -globin expression. This suggests that the interaction of cGATA-1 at the specialized TATA box is essential for the regulation of nucleosome assembled β -globin genes by helping to establish a basal initiation complex whose activity is controlled by stage-specific factors. However, a mutation to a canonical TATA box, which eliminates cGATA-1 binding, still allows the gene to be activated in chromatin presumably as a result of its high affinity for TFIID (-30 TATA; lane 12), which is present in the RBC nuclear extracts during preincubation.

The -30 GATA box regulates erythroid-specific accessibility of β -globin chromatin

Because a minimal promoter is sufficient to form transcriptionally active β -globin chromatin, we wished to identify the individual activator proteins that conferred open promoter commitment during nucleosome assembly. For this purpose, RBC nuclear extracts were fractionated by a combination of DNA-cellulose and FPLC-Mono S chromatography. Initially, we analyzed a protein fraction that was highly enriched for cGATA-1 but also contained some NF-E4-binding activity (Fig. 5A, cGATA-1 FPLC fraction). Further analysis of this FPLC fraction by DNase protection and gel mobility-shift assays indicated that no other β -globin promoter-binding activities were present (data not shown). In this chromatin transcription experiment, increasing amounts of the cGATA-1 FPLC fraction were preincubated with β -globin genes before nucleosome assembly. As shown in Figure 5A, this protein fraction alone is capable of establishing an open promoter complex that is efficiently and accurately transcribed (lane 4) at $\sim 60\%$ of the level obtained with the 11-day RBC extract (lane 5).

Figure 5. The cGATA-1 FPLC protein fraction functions through a -30 GATA box to establish an open promoter complex on chromatin assembled β -globin genes. (A) An FPLC protein fraction enriched in cGATA-1 activates β -globin chromatin templates only from -30 GATA-containing promoters. Chromatographically fractionated cGATA-1 protein was preincubated with β -globin genes before nucleosome assembly with 0, 1, 2, and 4 footprinting units (fpu) on β -globin promoters containing either -30 GATA boxes (lanes 1–4) or promoters mutated to -30 TATA boxes (lanes 7–10). These genes were also preincubated with 15 μ l of 37 mg/ml 11-day RBC nuclear proteins as shown in lane 5 (-30 GATA) and lane 11 (-30 TATA). β -Globin promoters containing a GATA site at -30 were transcriptionally active when preincubated with appropriate concentrations of the cGATA-1 FPLC fraction (lane 4), whereas templates containing the canonical TATA site at -30 remained inactive in the presence of this protein fraction (lanes 7–10). (B) Recombinant human TBP activates β -globin chromatin templates only from -30 TATA-containing promoters. Human TBP was preincubated with β -globin genes before chromatin assembly in the following amounts: 0, 1, 2.5, and 5 fpu on β -globin promoters containing either -30 GATA boxes (lanes 1–4) or promoters mutated to -30 TATA boxes (lanes 6–9). These promoters were also preincubated with 15 μ l of 37 mg/ml 11-day RBC nuclear proteins as shown in lane 5 (-30 GATA) and lane 10 (-30 TATA). Human TBP formed transcriptionally active β -globin templates with -30 TATA promoters, but -30 GATA promoters were repressed by nucleosome assembly in the presence of equal concentrations of hTBP.

This result substantiates the observation that occupancy of the -30 GATA site is critical for chromatin activation, as shown previously by the analysis of β -globin promoter deletions (Fig. 4B). Moreover, this also implicates a role for the NF-E4 protein in this process as the level of transcription obtained with the FPLC fraction more closely approximates that of promoters bound by both cGATA-1 and NF-E4 (-58 ; Fig. 4B, lane 6) than by cGATA-1 alone (-39 ; Fig. 4B, lane 8).

The specialized TATA box (-30 GATA) has been shown to be required for efficient enhancer-dependent β -globin expression in transfected chick erythroid cells. Mutation to a canonical TATA sequence, which eliminates cGATA-1 binding but preserves TFIID function, selectively decreases the promoter response to the distal enhancer (Fong and Emerson 1992). Because the -30 GATA site is critical for erythroid-specific β -globin gene expression, we examined how conversion to a TATA sequence affects the ability of this gene to be activated when assembled into chromatin. As shown in Figure 5A, incubation of the β -globin TATA templates with increasing concentrations of the FPLC fraction before nucleosome formation fails to alleviate transcriptional repression (lanes 7–10) indicating that the binding of NF-E4, present in this fraction, is insufficient if cGATA-1 cannot interact at -30 . However, preincubation with 11-day RBC proteins, which contain TFIID, generates β -globin TATA chromatin templates that are actively expressed (lane 11). To test this further, cloned TBP was analyzed for its ability to activate nucleosome-repressed β -globin genes containing either -30 GATA or TATA sequences. As shown in Figure 5B, chromatin templates assembled in the presence of TBP are transcriptionally active only when the -30 GATA sequence is mutated to a canonical TATA box (lanes 6–9). TBP cannot generate an active chromatin structure on β -globin genes containing the specialized -30 GATA box (lanes 1–4).

These experiments demonstrate that the DNA sequence of the -30 site is critical in determining which proteins are required to activate nucleosomal templates. This has important functional consequences for tissue-specific gene expression as the presence of a noncanonical TATA sequence in the β -globin promoter ensures that chromatin accessibility is regulated by the interaction of an erythroid protein, cGATA-1, rather than a general factor, TFIID.

cGATA-1 functions with a stage-specific factor to generate transcriptionally active β -globin chromatin

The importance of the -30 GATA box in the tissue-specific regulation of chromatin assembled β -globin genes led us to examine the role of purified GATA-1 in this process. Initially, we used affinity-purified cGATA-1 from 11-day chick RBCs and recombinant mGATA-1 in attempts to activate transcription from β -globin genes reconstituted into nucleosomes but were unsuccessful even over a wide range of protein concentrations (data not shown). On the basis of these results, we reasoned that another protein may be required to

function with GATA-1 and that a likely candidate was NF-E4. This factor is stage specific, binds next to cGATA-1 on the β -globin promoter, and mutations in its binding site result in deregulation of the β - ϵ enhancer (Gallarda et al. 1989; Foley and Engel 1992). We prepared affinity-purified NF-E4 from 11-day chick RBCs and analyzed its ability to activate transcription from β -globin chromatin templates either alone or in combination with GATA-1. As shown in Figure 6, purified mGATA-1 (lane 5), cGATA-1 (lane 6), or NF-E4 (lane 7) when assayed individually is unable to activate expression of chromatin-assembled β -globin genes, relative to genes assembled in the absence of protein (lane 2). However, efficient activation, which is equivalent to that obtained using RBC nuclear proteins (lane 3), occurs when either mGATA-1 or cGATA-1 is combined with NF-E4 (lanes 8–10). This activation, like that of the RBC nuclear proteins (lane 4), is dependent on RNA polymerase II, as shown by its sensitivity to low levels of α -amanitin (lane

Figure 6. GATA-1 and a stage-specific protein are required for developmentally regulated transcription of β -globin chromatin. Recombinant mGATA-1 or affinity-purified cGATA-1 and NF-E4 from 11-day chick RBCs were tested for their ability to activate β -globin transcription from chromatin templates. These proteins were analyzed individually or in combination and 11-day RBC nuclear proteins were used as a control. β -Globin genes were preincubated under the following conditions before chromatin assembly and transcription: (Lane 1) unassembled DNA; (lane 2) nucleosome-assembled DNA without protein preincubation; (lane 3) preincubation with 15 μ l of 37 mg/ml 11-day RBC nuclear proteins; (lane 4) plus 100 ng/ml α -amanitin; (lane 5) 5 fpu of recombinant mGATA-1; (lane 6) affinity-purified cGATA-1; (lane 7) or affinity-purified NF-E4; (lane 8) 5 fpu each of mGATA-1 and NF-E4; (lane 9) 3 fpu each of mGATA-1 and NF-E4; (lane 10) 5 fpu each of cGATA-1 and NF-E4; (lane 11) 5 fpu each of mGATA-1 and NF-E4 plus 100 ng/ml α -amanitin; (lane 12) 5 fpu each of mGATA-1 and NF-E4 with β -globin genes containing TATA promoters; (lane 13) 5 fpu each of mGATA-1 and NF-E4 added after nucleosome assembly; (lane 14) 5 fpu each of hTBP and NF-E4; and (lane 15) 5 fpu each of mGATA-1 and AP-1.

11). β -Globin transcription resulting from the binding of mGATA-1 and NF-E4 to the promoter requires a -30 GATA box, as no expression is observed using templates containing a -30 TATA sequence (lane 12). This confirms the requirement for the direct interaction of mGATA-1 with the critical -30 site and argues against the possibility that NF-E4 might "tether" mGATA-1 in this vicinity by protein-protein contacts alone. The combination of mGATA-1 or cGATA-1 with NF-E4 appears to be specific, as other transcriptional factors, such as hTBP or AP-1, cannot activate β -globin expression when combined with either NF-E4 (lane 14) or mGATA-1 (lane 15), respectively. However, the effects of the other stage-specific proteins, β -CTF and β -AP2, on β -globin chromatin activation in the presence of GATA-1 have not been examined. Order-of-addition experiments demonstrate that mGATA-1 and NF-E4 cannot activate transcription if they are added after chromatin assembly (lane 13), indicating that their DNA-binding sites are not recognized efficiently when packaged into a nucleosome.

Discussion

The tissue- and developmental stage-specific expression of the chick β -globin gene is correlated with highly regulated changes in chromatin structure. We have examined the nature of changes that occur on the local control regions by focusing on the activation of the β -globin promoter when assembled into chromatin. Our experiments take advantage of *in vitro* systems that allow the efficient reconstitution of β -globin genes into nucleosomal structures using extracts from *Xenopus* eggs and transcriptional analysis using staged erythroid extracts. The premise in these studies is that nucleosome assembly results in transcriptional repression as a result of an inaccessibility of the promoter to regulatory proteins. Chromatin can be "remodeled" into an active structure by the interaction of specific DNA-binding proteins, which prevents nucleosome formation on critical control regions. This structural change generates transcriptionally active templates that are accessible to RNA polymerase II and other required factors. The problems presented by chromatin inaccessibility to transcription by RNA polymerases II and III have been reviewed recently (Kornberg and Lorch 1991; Hayes and Wolffe 1992; Adams and Workman 1993; Struhl 1993). Following this rationale, we present evidence that the assembly of the chick β -globin gene into actively expressed chromatin is dependent on the prebinding of nuclear proteins from extracts of definitive RBCs that maximally transcribe the endogenous β -globin gene. Proteins derived from other stages of erythroid development or from non-erythroid cells fail to generate active β -globin chromatin templates. Thus, tissue- and developmental stage-specificity of chromatin activation are reproduced in this *in vitro* system.

An analysis of promoter deletions demonstrates that only a minimal DNA sequence (-58) is necessary to produce transcriptionally active chromatin. This se-

quence encodes the binding sites for an erythroid stage-specific factor, NF-E4, and an erythroid-restricted factor, cGATA-1, which is present throughout RBC development. Promoters containing only the -30 GATA site (-39) are capable of correctly initiated basal level transcription, which is significantly increased by sequences that bind the stage-specific activators NF-E4 (-50), β -CTF (-70), and β -AP2 (-90). This indicates that the initiation complex formed at -30 is sufficient to determine an accurate transcriptional start site but that developmentally regulated proteins control the efficiency of this complex. We show that incubation of both cGATA-1 and NF-E4 with β -globin genes before nucleosome assembly is required to generate transcriptionally active chromatin templates. This is demonstrated using FPLC fractions containing both cGATA-1 and NF-E4 or affinity-purified proteins. In addition, recombinant mGATA-1 can replace cGATA-1 functionally. In these experiments, the interaction of cGATA-1 (or mGATA-1) with the -30 GATA site is critical for chromatin activation, as β -globin genes mutated to a canonical TATA box remain transcriptionally inactive in the presence of cGATA-1 and NF-E4. Instead, these promoters can be activated by the interaction of recombinant TBP before nucleosome assembly. However, TBP cannot replace cGATA-1 to activate -30 GATA promoters even in the presence of NF-E4.

It is interesting that preincubation with both cGATA-1 and NF-E4 is required to activate chromatin-assembled β -globin genes, as occupancy of the -30 site by TBP alone is sufficient to derepress β -globin genes with TATA promoters. This is similar to earlier experiments demonstrating that the interaction of either TFIID or recombinant TBP with the -30 TATA box in the adenovirus major late promoter (MLP) can activate chromatin-repressed transcription (Meisterernst et al. 1990; Workman and Roeder 1987). TFIID also functions with upstream activators to increase the transcriptional efficiency of nucleosome-assembled genes. A ubiquitous cellular factor (USF) binds to chromatin templates only with derepression by TFIID, and stimulates transcription by stabilizing or increasing the rate of TFIID interaction (Workman et al. 1990; Meisterernst et al. 1990). Similarly, the *Drosophila hsp70* gene requires the initial binding of TFIID for chromatin derepression as this enables the heat shock factor (HSF) in *Drosophila* extracts to interact with the promoter after nucleosome assembly and activate transcription. If chromatin is formed in the absence of TFIID, the heat shock promoter remains inaccessible to HSF binding and is transcriptionally inactive (Becker et al. 1991).

In contrast to the requisite TFIID binding in the preceding examples, the GAL4-VP16 chimeric protein provides the initial chromatin derepression of a synthetic promoter when bound to multiple Gal4 sites. TFIID added after nucleosomal assembly can generate, along with other basal transcription factors, an active initiation complex on GAL4-VP16-derepressed templates (Workman et al. 1991). In addition, nucleosomal gel-shift assays have shown that transcriptional activators have

different capabilities to interact with their binding sites within a nucleosomal structure. These studies corroborate the functional observations made with the coupled chromatin assembly/transcription systems described above. Specifically, the GAL4 protein can recognize its DNA-binding site within a nucleosome, whereas the HSF can bind only when its site is made accessible by TFIID (Taylor et al. 1991).

Other DNA-binding proteins interact with chromatin in a manner that has not been related directly to TFIID binding at the -30 promoter site. Well-studied examples include the glucocorticoid receptor (GR) and its interactions with various transcriptional activators that have different capacities to bind nucleosomal structures. The ability of the GR to bind its regulatory element (GRE) when it is packaged in a nucleosomal core complex has been documented both *in vivo* and by *in vitro* reconstitutions (Perlmann and Wrangé 1988; Pina et al. 1990; Archer et al. 1991; Perlmann 1992). In one study using the mouse mammary tumor virus (MMTV) promoter, the GR interacted efficiently with its cognate-binding site when reconstituted into a nucleosome, whereas the transcription factor NF1 could not recognize its binding sequence in chromatin unless the GR was previously bound (Pina et al. 1990). Similarly, hormone-regulated expression of the rat tyrosine aminotransferase (TAT) gene is achieved, in part, by the interaction of the transcriptional activator HNF5, a liver-specific protein, with the TAT promoter in a GR-dependent manner. *In vivo* footprinting studies revealed that the GR binds on exposure to hormone and disrupts the nucleosomal structure of the TAT promoter in a hit-and-run mechanism that leaves the site accessible to HNF5 (Reik et al. 1991; Rigaud et al. 1991). Thus, in both the MMTV and TAT systems, the requirement of the GR to bind chromatin initially establishes open promoter complexes that are accessible to transcription factors and imposes stringent hormonal control on gene expression.

In the β -globin system, we show that developmental control of transcription is attributable to the dual requirement of an erythroid-restricted protein and a stage-specific factor to activate chromatin. The potentiation activity of TFIID, GAL4-VP16, or the glucocorticoid receptor is not comparable directly to GATA-1 binding at the specialized -30 site of the β -globin gene as this protein cannot act alone to generate an open promoter complex nor does it enable the interaction of other factors after chromatin assembly. The necessity of cofactors for cGATA-1 activity may be a common feature in the regulation of erythroid-expressed genes, as it would enable this protein to function in a context- and developmentally-dependent manner. For example, consensus-binding sites for cGATA-1 exist in the promoters and enhancers of most globin genes (Evans et al. 1988; Plumb et al. 1989), as well as in the erythroid locus control region (Talbot et al. 1990; Talbot and Grosveld 1991), yet these genes are stage-specifically expressed. Because cGATA-1 is present throughout RBC development (Yamamoto et al. 1990), it may combine with stage-specific proteins to restrict its activity on a particular gene to an appropriate

time. This is exemplified by the observation that the two embryonic globin genes, ρ and ϵ , contain -30 GATA boxes but are not expressed at the same time as the β -globin gene, even though cGATA-1 is present at both stages of development. A possible regulatory mechanism to account for this is that some -30 GATA sites may be inaccessible as a result of nucleosome blockage at certain times in development. Alternatively, cGATA-1 may interact with these -30 sites but be nonfunctional in the absence of factors specific to either the primitive or definitive RBC lineage. An example of such a factor is NF-E4, which is a strong transcriptional activator of β -globin expression *in vitro* (Emerson et al. 1989). This protein is present only in definitive RBCs and has been implicated, with β -CTF, in regulating the switch from ϵ - to β -globin gene expression by altering the affinity of the shared β - ϵ enhancer to favor the β -globin promoter (Gallarda et al. 1989; Foley and Engel 1992). The requirement of a coactivator, like NF-E4, to function with cGATA-1 in activating β -globin chromatin expression is particularly advantageous for erythroid regulation, as both tissue and developmental specificities are imparted by these two proteins. It should be mentioned that our data do not exclude the possibility that another stage-specific factor, β -CTF, could also combine with cGATA-1 to activate β -globin expression from chromatin templates.

Previous studies have shown that the binding of cGATA-1 to a specialized TATA box (GATA) in the β -globin promoter is functionally significant, as it regulates the ability of this control region to interact with the distal 3' enhancer. A mutation to a canonical TATA box preserves promoter activity by TFIID but eliminates cGATA-1 binding and severely decreases the enhancer response. Both TFIID and cGATA-1 have distinct roles in β -globin transcriptional activation and function at different times through the -30 GATA site. cGATA-1 is proposed to bind initially the enhancer and the -30 GATA box to establish an interaction between these two control regions, perhaps in combination with stage-specific proteins, as with NF-E4 and β -CTF. TFIID (TBP) then complexes with adaptor proteins and displaces cGATA-1 from the -30 site to form an active initiation complex (Fong and Emerson 1992). A model involving the stage-specific factors in enhancer recruitment was originally proposed by Engel and co-workers (Gallarda et al. 1989). Thus, there is a functional interplay between cGATA-1, NF-E4, TFIID, and adaptor proteins in the developmental control of β -globin gene expression. We propose that the tissue- and stage-specific factors, cGATA-1 and NF-E4, have crucial roles in the regulation of chromatin accessibility in the promoter and its interaction with the distal 3' enhancer. Once these conditions are established, the general factor TFIID activates transcription by forming an initiation complex at the -30 GATA box. The occupancy of this specialized TATA box by either cGATA-1 or TFIID is modulated by the adaptor proteins.

It is interesting that both cGATA-1 and mGATA-1 are equally functional with NF-E4 in the derepression of chromatin-assembled β -globin genes. The avian and

mammalian GATA-1 proteins share significant homology in their DNA-binding domains, but are widely divergent in their transcriptional activation regions (Martin and Orkin 1990; Yamamoto et al. 1990). The requirement for acidic activation domains for efficient transcription of chromatin templates was demonstrated recently using derivatives of the GAL4 protein (Workman et al. 1991). Future studies will attempt to define the regions of the GATA-1 protein that are critical for the activation of nucleosome repressed genes as well as to examine the specificity of other tissue-restricted members (Wilson et al. 1990; Yamamoto et al. 1990; Ko et al. 1991) of the GATA family in this process.

Materials and methods

Plasmid constructions

The chick β -globin gene used in these studies, pUC18ABC/ Δ 1, has been described previously (Emerson et al. 1989) and is the parental plasmid for all other constructs. This genomic clone contains the promoter region of the adult β -globin gene from -407, the entire protein-coding region including a 17-bp insertion of polylinker sequences at +46, and 1.5 kb of 3'-flanking sequences including the β - ϵ gene enhancer. β -Globin genes mutated at the -30 GATA site to a canonical TATA box (-30 TATA) or to a nonbinding mutation GAGGA (-30 GG) were constructed as previously described (Fong and Emerson 1992). β -Globin templates deleted from the 5' end of the promoter to -120, -58, and -39 are described in Emerson et al. (1989). A β -globin construct containing a point mutation AAGTGGa at -50 (-NF-E4) was the generous gift of the late Dr. Joanne M. Nickol.

Chromatin assembly extracts

Unactivated egg extracts capable of chromatin assembly were prepared by the procedure of Murray and Kirschner (1989), with some modifications. Five to seven female *Xenopus laevis* (Nasco) were induced to ovulate by injection with human chorionic gonadotropin (Sigma). Large quantities of eggs were laid overnight in $1 \times$ MMR [5 mM HEPES (pH 7.8), 100 mM NaCl, 2 mM KCl, 1 mM MgSO_4 , 2 mM CaCl_2 , 0.1 mM EDTA] and were collected, dejellied with 2% cysteine (pH 7.8), washed, and lysed by centrifugation essentially as described, but with the following alterations: The eggs were not activated by electrical stimulation or incubation in Ca^{2+} ; therefore, the incubation in H_2O before dejellied was omitted. The extract buffer, XBII [100 mM KCl, 4 mM MgCl_2 , 10 mM K-HEPES (pH 7.2), 100 mM sucrose, 0.1 mM EGTA] was used for all further washes instead of the published XB buffer. Cycloheximide (10 $\mu\text{g}/\text{ml}$) and DTT to 1 mM concentration were added, and cytochalasin B and Versilube were omitted during the final wash with this buffer. Lysis by centrifugation, collection of the clarified, crude cytoplasm, addition of the energy mix (20 $\times$ concentration: 20 mM MgCl_2 , 20 mM ATP, 150 mM creatine phosphate, and 20 mM DTT) were as described with the addition of cytochalasin B (Sigma) to a final concentration of 10 $\mu\text{g}/\text{ml}$. After centrifugation, the clarified egg cytoplasm was fractionated further by spinning at 200,000g as described by Wilson and Newport (1988) except that a Beckman SW55 rotor was used to accommodate the larger volume of egg extract. The membrane-free soluble fraction was collected by side puncture, and 100- μl aliquots were quickly frozen in liquid nitrogen and then stored at -100°C . This high-speed supernatant was used as a source of histones and nucleosome assembly factors in all of the chromatin assembly assays.

Preparation of extracts and purification of proteins

Nuclear protein extracts from 5-day, 11-day, and adult RBCs, as well as chick brain, were prepared as described previously (Emerson et al. 1987). Transcriptionally active extracts from 11 day chick RBC were obtained according to Emerson et al. (1989). Chromatography of nuclear extracts on calf thymus DNA cellulose (Sigma) and elution with 0.25 M $(\text{NH}_4)_2\text{SO}_4$ (DC fxn) yielded high-affinity DNA-binding proteins (Emerson et al. 1987). Purification of the cGATA-1 and NF-E4 proteins from 11-day RBC nuclear extracts by DNA cellulose and sequence-specific affinity chromatography was according to the procedure of Kadonaga and Tjian (1986). Further purification of DNA-cellulose fractionated proteins was achieved using a 0.05–1 M KCl gradient elution [in 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM DTT] of a Mono S ion-exchange column by FPLC chromatography (Pharmacia). This column was run at a flow rate of 1 ml/min, and 0.5-ml fractions were collected. The peak of cGATA-1-binding activity coincided with that of NF-E4 (fractions 16–20), although no other specific or nonspecific binding activity was detected in these fractions. Recombinant mouse GATA-1 protein (pETmGATA-1), a kind gift of Drs. Russell Kaufman and Scott Langdon (Duke University Medical Center, Durham, NC), was expressed in BL21(DE3)pLysS cells and purified by chromatography on DNA-cellulose and sequence-specific affinity columns. Recombinant human TBP was obtained from Promega.

In vitro transcription of nucleosome-assembled DNA templates

β -Globin DNA templates (50–100 ng) plus nonspecific DNA (0.40–0.45 μg of sCos1 vector, described in Evans et al. 1989) were incubated with nuclear extracts, purified proteins, or buffer, as indicated, for 20 min at room temperature before chromatin assembly. After this preincubation step, *Xenopus* egg extracts were added to assemble chromatin at $t = 0$. Before this time, the *Xenopus* extracts were quick-thawed and centrifuged in a 4°C microcentrifuge for 2 min, and the clarified supernatant was incubated on ice for 10 min with poly[d(I-C)] (Boehringer Mannheim) at a final concentration of 15 ng/ μl ; additional ATP (2.8 mM) and MgCl_2 (4.7 mM) were also added. Nucleosome assembly occurred over a 1-hour period at 22°C . At $t = 60$ min, transcription of the assembled DNA templates was initiated by addition of transcription extracts prepared from 11-day chick RBCs and adjusted to standard transcription conditions as described previously (Emerson et al. 1989) with an NTP/salts/energy-generating mix to a total volume of 80 μl . After a 60-min incubation at 30°C , the reaction products were analyzed by S1 nuclease and gel electrophoresis (Emerson et al. 1989). The relative amounts of RNA in each reaction were determined by scanning several autoradiogram exposures of each transcription experiment followed by image analysis using the National Institutes of Health (NIH) Image 1.43 program.

Chromatin structural analysis

Nucleosome assembly on the β -globin template was assessed by DNA supercoiling and staphylococcal nuclease digestion after incubation with the *Xenopus* egg extract. β -Globin DNA (0.5 μg) was preincubated with buffer or RBC nuclear extracts or purified proteins as described above and then reconstituted into nucleosomes by incubation with 1/10 volume of AB buffer [0.2 M HEPES (pH 7.9), 0.5 M KCl, 50 mM MgCl_2 , 30 mM ATP, 10 mM DTT, and 1 mg/ml BSA], XBII buffer, and increasing amounts of *Xenopus* egg extract in a total volume of 40 μl for 1 hr at 22°C . For supercoiling analysis, reactions were stopped by the addition of EDTA, SDS, and proteinase K to final concentrations of

7.5 mM, 0.1 mM, and 1 mg/ml, respectively, and incubated at 37°C for 15 min. The samples were then extracted with phenol/chloroform and chloroform, ethanol precipitated, and analyzed by electrophoresis on a 0.8% agarose/TBE gel at 55 V for 16 hr. For analysis by staphylococcal nuclease, reactions were diluted with an equal volume of SM2 buffer [500 mM sucrose, 80 mM KCl, 20 mM HEPES (pH 7.5), 3.5 mM ATP, and 6 mM CaCl₂] after nucleosome assembly and digested with 150 units of staphylococcal nuclease (Boehringer Mannheim) for 2.5, 5, 15, and 30 min at room temperature. Similarly, limit digests with staphylococcal nuclease were performed for 2 hr at room temperature. The digestions were stopped by the addition of 20 mM EDTA and 2 mM EGTA, final concentrations, and treated with 1 mg/ml of RNase A (Sigma) for 30 min at 37°C. After the addition of 8 µg of glycogen (Calbiochem) as carrier, the samples were digested with 1 mg/ml of proteinase K (Merck) in an equal volume of SM3 buffer [0.2% SDS, 8 mM EDTA, 20 mM Tris (pH 8.0)] for 1 hr at 37°C. The DNA was purified by extraction with phenol/chloroform and chloroform, ethanol precipitated, and analyzed by electrophoresis on a 1.5% agarose-TBE gel at 47 Volts for 16 hr. Southern blot analysis of the agarose gel was carried out by blotting with a Stratagene (La Jolla, CA) Posiblott pressure blotter onto GeneScreen Plus (New England Nuclear) and hybridization and washing as described by Church and Gilbert (1984). The ³²P-radiolabeled 123-bp ladder and linearized pUC18ABC/Δ1 DNA were used as probes either as a mixture or sequentially. Sucrose gradient purification of mononucleosome populations was performed after staphylococcal nuclease digestions scaled up for the analysis of 10 µg of DNA and carried out for 2 hr at room temperature. An aliquot of this digest containing 200 ng of β-globin DNA was removed and analyzed by electrophoresis and Southern blotting as described above. The remainder of the protein-DNA mixture was dialyzed for 6 hr against the buffer used in the sucrose gradients [100 mM NaCl, 5 mM HEPES (pH 7.9), 0.2 mM EGTA] and then fractionated on a 5–29% sucrose gradient as described previously (Shimamura et al. 1988) but run for 16 hr at 30K rpm in a Beckman SW41 rotor. Sucrose gradients were fractionated with an Isco density gradient fractionator (Lincoln, NE) and 300-µl fractions collected. A portion of every other fraction (2–40) was electrophoresed on a 1.2% TBE-agarose gel in the presence of 0.2% SDS and Southern blot analysis was carried out as described to determine the relative positions of the mononucleosome populations.

MspI cleavage sensitivity

The exact conditions for preincubation and chromatin assembly of β-globin DNA templates before transcription were followed for the MspI digestion analyses. All extracts and partially purified proteins were prepared as described above. After the 1-hr chromatin assembly period, the reconstituted complexes were assayed for MspI accessibility essentially as described by Emerson and Felsenfeld (1984). The assembly reactions were made 10 mM in MgCl₂ and 10 units of MspI were added to each and incubated at 37°C for 30 min. The samples were then digested with RNase A (10 µg) for 15 min followed by proteinase K for an additional 10 min at 37°C. The DNA samples were purified by phenol/chloroform extraction (1 : 1), precipitated with ethanol/ammonium acetate and resuspended in 15 µl of water before electrophoresis through a 1.8% agarose-TBE gel for 400 V/hr. Southern blot analysis was carried out as described above with a gel-isolated fragment from the –109 to –224 region of the chick β-globin promoter as probe. As a control for hybridization specificity and specific promoter fragment release, the 110- and 123-bp bands of an MspI digest of pBR322 were gel isolated,

³²P-labeled, and used as probe for the same DNA blots after the blots were stripped according to the suggested procedure in the GeneScreen Plus (New England Nuclear) manual.

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The erythroid protein cGATA-1 functions with a stage-specific factor to activate transcription of chromatin-assembled beta-globin genes.

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