Recent studies indicate that a purified protein, activin A, belongs to the transforming growth factor β (TGF-β) superfamily. Similar to TGF-β, activin A can have different biologic activities, depending on the target tissues. We used recombinant activin A to demonstrate a possible regulatory role of this protein in modulating human erythroid differentiation in the human erythroid cell line, K562. Using genomic probes containing the second exon of α, β, γ, and e globins, relative abundance of various types of globin transcripts in untreated and activin-treated K562 cells was examined with S, nuclear analysis. Despite considerable homology amongst various globin sequences, these globin probes were highly specific for their unique mRNA species in the analyses. It was shown that the abundance of specific globin probe fragments for γ and e globins (209 nucleotides) as well as α (180 nucleotides), which were protected from S, digestion, increased many fold in K562 cells treated with activin A. In contrast, there were no specific transcripts of β globin detected in either the control or activin-treated cells. The increases in the level of fetal and embryonic β-like and e globin transcripts also confirmed earlier studies of Northern and slot-blot analyses using globin cDNA as probes. In addition, nuclear run-off transcription assay using isolated nuclei indicated that most of the increase in the globin transcripts after activin treatment could be attributed to the stimulation of transcription rate for globin genes. Transient transfection assays also provide evidence that activin A significantly stimulated transcriptional activity of an e globin promoter in K562, but not in the nonerythroid Chinese hamster ovary cells. Therefore, it was concluded that activin A exerts its effects on globin gene expression at the level of transcription in erythroid cells.

**MATERIALS AND METHODS**

**Cell cultures.** Stock cultures of the K562 cell line were grown in RPMI 1640 medium supplemented with 50 IU/mL of penicillin, 50 μg/mL of streptomycin and 15% fetal calf serum, as previously described.12 Cell cultures at density of 1 to 2 × 10^6/mL were incubated with recombinant human activin (generous gift from Dr R. Schwall, Genentech, Inc, South San Francisco, CA) stock solution added to the specified final concentration. The recombinant activin A (ie, β,β, in this study) was purified from spent culture medium of mammalian kidney cells after transfection with a plasmid containing the entire coding region of the β, subunit under the control of a cytomegalovirus promoter.

**RNA preparation.** Total RNA was prepared from 1 to 3 × 10^7 K562 cells by the guanidinium isothiocyanate method.10,11 After being washed three times with phosphate-buffered saline, the cells were lysed in 4 mol/L guanidinium isothiocyanate, 20 mmol/L sodium acetate, pH 5.2 containing 0.1 mmol/L dithiothreitol and 0.5% N-lauryl sarcosine (Sarkosyl; Sigma, St Louis, MO); they were then sheared through a 22-gauge needle. Samples were spun in a microfuge to pellet debris, the supernatant was recovered, and cesium chloride was added to a concentration of 0.4 g/mL. This mixture was then layered over a cushion of 5.7 mol/L CsCl and centrifuged at 35,000 rpm in a Beckman SW 50.1 Ti rotor (Beckman, Palo Alto, CA) for 16 to 20 hours. After centrifugation, the RNA pellet was dissolved in H_2O; NaOAc was added, and the sample was extracted with phenol/chloroform. RNA was then precipitated with ethanol and dissolved in H_2O.

**Northern and slot-blot analyses.** For Northern analyses, 12.5 μg of total RNA was loaded on 1% agarose gel containing 1.1 mol/L formaldehyde in 20 mmol/L 3-(N-morpholino) propanesulfonic acid, 5 mmol/L NaOAc, 1 mmol/L EDTA, pH 8.0 buffer. After

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electrophoresis at 175 V for 2.5 hours, samples were transferred to nitrocellulose. The filters were then baked at 80°C for 2 hours under vacuum. Prehybridization of the filters took place at 42°C overnight in 56% formamide, 5X Denhardt's solution, 5X 1,4-piperazine-diethanesulfonic acid (PIPES), 0.2% sodium dodecyl sulfate (SDS), 0.2% NaPPi, and 100 μg/mL salmon sperm DNA. The filters were then hybridized overnight at 42°C with 2 to 5 × 10^6 cpm/mL of nick-translated probe in the same prehybridization solution except that 1X Denhardt's solution was used. After hybridization, the filters were soaked at room temperature for 15 minutes in 2X SSC (1X SSC is 0.15 mol/L sodium chloride, 0.025 mol/L sodium citrate) with 0.1% SDS and then washed in subsequently decreasing concentrations of SSC as follows: 2X SSC at 42°C for 1 hour, 1X SSC at 42°C for 1 hour, 1X SSC at 58°C for 45 minutes, 0.2X SSC at 58°C for 45 minutes, 0.2X SSC at 68°C for 45 minutes, and 0.1X SSC at 68°C for 45 minutes. Afterward, the filters were rinsed at room temperature in 1X SSC without SDS and subsequently decreasing concentrations of SSC as follows: 2X SSC (1X SSC is 0.15 mol/L sodium chloride, 0.025 mol/L sodium citrate) with 0.1% SDS and then washed in buffer C (1 mmol/L Tris-HCl, pH 7.6, 25 mmol/L KCl, 0.9 mmol/L MgCl_2, 0.9 mmol/L CaCl_2, 0.14 mmol/L spermidine), once in buffer C plus 1 mmol/L phenylmethylsulfonyl fluoride, and lysed in a Dounce homogenizer (Baxter Sci. Products, McGaw Park, IL). Nuclei were pelleted, washed twice in buffer C, and resuspended at 40 μg/mL. Afterward, these isolated nuclei were used in the following in vitro transcription assay. The standard labeling assay described by Hofer and Darnell used 125 μCi of [α-32P]UTP (3,000 Ci/mmol; New England Nuclear) per 10^6 nuclei. Incorporation of [32P]UTP into RNA was monitored by counting the radioactivity in the RNA products. The specific activity of the labeled RNA was determined by hybridization with the 5' noncoding region of the human globin gene. The probes were bound to nitrocellulose by the method of Kafatos et al. After denaturation in 0.2 N NaOH at 100°C for 5 minutes and neutralization with 2 mmol/L HEPES. Approximately 2.5 μg of DNA was bound per dot. Hybridizations were performed as described above. After hybridization, the samples were washed with 2X SSC and then treated with 2.5 μg/mL of RNase A and 5 μg/mL of RNase T, at 37°C for 60 minutes. The RNase-resistant hybrids were quantitated by scintillation counting.

**Transfection and chloramphenicol acetyltransferase (CAT) assay.** Approximately 2 × 10^6 rapidly growing K562 cells were washed and resuspended in 0.5 mL HEPES-buffered saline containing the pGLCAT and the pRSVLUC plasmids (gifts from Dr A. Schechter at NIH, Bethesda, MD, and Dr A. McLachlan at Scripps Clinic). After incubation at room temperature for 10 minutes, the mixture was electroporated with 1 pulse from a Bio-Rad (Richmond, CA) “Gene Pulser” set to deliver 220 V for 18 to 19 milliseconds. In other experiments, the monolayer Chinese hamster ovary cells were transfected with standard calcium phosphate precipitation methods. The cells were then allowed to stand at room temperature for another 10 minutes before being divided and cultured in different media. In the presence and absence of activin A for 3 days, CAT assays were performed by incubating cellular extracts with 32C-chloramphenicol (Amersham, Arlington Heights, IL) and acetyl CoA in a manner similar to that previously described. Within each cell type, CAT activity for each plasmid was normalized to luciferase activity of the internal control plasmid pRSVLUC. For assay for the CAT activity, cell extracts obtained from transfected cells were first heated at 65°C for 10 minutes, a step important for CAT enzyme stability in K562 cell extracts. Then approximately 20 μL of extract was mixed with 66 μL of 0.25 mol/L Tris-HCl (pH 7.8), 20 μL of 4 mmol/L acetyl CoA, water, and 0.5 μCi of [32C]-chloramphenicol (50 mCi/mmol; New England Nuclear) and the reaction was performed at 37°C for 30 minutes. Under these conditions, the activity of the CAT enzyme was linear for greater than 3 hours. The reaction was then stopped and the chloramphenicol was extracted with 1 mL ethyl acetate, and spotted on a silica gel thin-layer plate to separate the chloramphenicol from its acetylated derivatives. Migration was in chloroform-methanol (95:5) for 20 minutes. After autoradiography overnight, the spots in thin layer chromatography plates were cut out and counted to quantitate the amount of chloramphenicol converted into its 1- and 3-acetyl form. CAT activity was expressed as the percentage of conversion of chloramphenicol into its 1- and 3-acetyl form.
RESULTS

Northern and slot-blot analyses for globin transcripts. To examine the effect of activin A on the expression of globin genes, RNAs prepared from control and activin-treated K562 cells were subjected to Northern analyses using various specific human globin cDNA probes. Figure 1 shows hybridization experiments in which equal quantities of RNAs were used for hybridization with α globin and actin probes. Hybridization of RNAs with α globin cDNA indicates a significant increase of globin transcripts (600 to 700 nucleotides in size) in the activin-treated K562 cells. Similarly, the use of γ and ε globin cDNA probes also demonstrate a similar enhancing effect of activin A on the levels of mRNA contents for these β-like globin genes (picture not shown). In other experiments, slot-blot analysis was performed to compare the expression of various globin transcripts, using RNAs corresponding to 1 x 10⁶ cells per slot and the same hybridization and washing conditions (Fig 2). With the use of cDNA probes for various globin transcripts, these slot-blot experiments confirmed that there was a significant increase in transcripts for α, γ, ζ, and ε globin in the K562 cells after incubation with activin A; but no expression of β globin mRNA was detected in either the control or activin-treated cells. Hybridization with the actin probe (as an internal control) showed that there were equal amounts of RNA in the control and activin-treated samples (Fig 2). When K562 cells were incubated with hemin, the increases in α, γ, ζ, and ε globin mRNAs were also noted (hybridization blot not shown) and the results correspond to the data previously reported. These studies indicate that activin causes a great increase in the contents of globin mRNA.

Accumulation of various globin transcripts. To quantitate the effect of activin A on the expression of various globin genes in K562 cells, we then measured the relative abundance of α, β, γ, and ε globin mRNAs in control and activin-induced samples by S, nuclease analyses using highly specific genomic probes. As shown in Figs 3 and 4, the activation of various types of globin genes in K562 cells after incubation with different concentrations of activin A was examined. The organization of the probes used is shown in Figs 3B and 4B. Exon 2 of the α, and α₂ human globin genes has the same nucleotide sequence and has a HindIII site 180 nucleotides downstream from the 5' end of this exon. Therefore, a probe fragment of 180 nucleotides...
on the noncoding strand would be expected to be protected from $S_i$ nuclease digestion by correctly spliced exons 2 from both the $\alpha_i$ and $\alpha_i$ globin genes in these experiments (Fig 3A). On the other hand, the exon 2 sequences of the $\epsilon$, $\gamma$, and $\beta$ globin genes are 77% to 87% conserved, and each contains a $BamHI$ site 209 nucleotides from the 5’ end. Correctly processed mRNAs of the $\beta$-like globin gene family would then protect probe fragments of 209 nucleotides from $S_i$ digestion of the noncoding strand of the probes (Fig 4A). The hybridization protocol used in these experiments utilized a 80% formamide solution and high-stringency conditions. In Figs 3 and 4, total RNA from nontreated and activin-induced K562 cells was hybridized to globin probes. After $S_i$ treatment and electrophoresis of the protected probe fragments, the gels were autoradiographed. Then, accumulation of globin mRNAs in K562 cells was examined by comparison with the relative abundance of the specific globin fragments in the autoradiographs. It was shown that the abundance of specific globin fragments for $\gamma$ and $\epsilon$ globins (209 nucleotides) as well as $\alpha$ globin (180 nucleotides) increased in K562 cells treated with increasing amounts of activin (Figs 3 and 4). Slot-blot hybridization with the probe for G3PD confirmed that there were equal amounts of RNA in the control and activin-treated samples (eg, Fig 3C) in these experiments. The results from four similar $S_i$ nuclease digestion experiments are summarized in Fig 5. It was shown that the levels of $\alpha$, $\gamma$, and $\epsilon$ globin transcripts were increased in a dose-responsive manner. Maximum induction of the increase of those globin transcripts was observed with activin A at approximately 100 ng/mL in the incubation medium (Fig 5). In addition, there were no specific transcripts of $\beta$ globin detected in either the control or activin-treated cells (Fig 4). The absence of cross-hybridization between the $\beta$ globin probe and other $\beta$-like globin mRNAs (ie, $\gamma$ and $\epsilon$ globin transcripts) in this analysis shows the specificity of each probe for its unique mRNA species, despite considerable homology among $\beta$, $\gamma$, and $\epsilon$ exon 2 sequences as discussed above. Furthermore, in the $S_i$ analyses of $\alpha$ globin mRNAs there was one additional band present above the expected 180-nucleotide band (Fig 3A). This band was reproducibly detected and its intensity was always proportional to that of the 180 nucleotide band. However, it is of uncertain significance and may represent an artifact of $S_i$ nuclease digestion. Thus, these data indicate that fetal ($\gamma$) and embryonic ($\epsilon$), as well as $\alpha$, globins are increased by incubation of cells with activin A, while no expression of $\beta$ globins is demonstrated in K562 cells.

Transcriptional activation of globin genes. Previously, a chemical inducer, hemin, was shown to cause accumulation of globin mRNA in K562 via a posttranscriptional regulation. To study the mechanism of the effect of activin A on the contents of globin gene transcripts, the following two experiments were performed. Using the nuclear runoff transcription assay, RNA labeled in isolated nuclei was hybridized to filter-bound DNA fragments containing various globin probes. It was found that the $\alpha$ and $\gamma$ globin genes were transcribed at a very low rate in uninduced K562
Fig 4. S, nuclease analyses of various β-like globin mRNA using specific β-like genomic probes. (A) K562 cells were incubated for 5 days with activin A at the concentrations specified in the figure. Then total RNA was isolated and analyzed for the abundance of specific mRNA with genomic probes for ε, γ, and β globins. The S, nuclease analyses were performed in a manner similar to that in Fig 3. Sample no. 1 in the gel represents the undigested probes used in each experiment; sample no. 2 did not include any RNA in the S, analyses, samples no. 3 through 8 contained 1 μg of total RNA that was isolated from K562 cells after incubation with activin A; and sample no. 9 in the gel indicates positions of various size standards, the pBR322/MspI digests. The sizes of these standards are identical to those in Fig 3. Equal amounts of RNA present in various samples were confirmed with slot-blot analyses as shown in Fig 3 (picture not included). (B) The organization of the probes used in the experiment is shown, with the symbols for exons, introns, and flanking sequences similar to those in Fig 3. The position of a BamHI site in exon 2 is indicated.

Fig 5. Effects of increasing concentrations of activin A on globin gene expression in K562 cells detected by S, nuclease analyses. K562 cells were incubated with various concentrations of activin A and total RNAs were analyzed with S, nuclease analyses with globin probes specific for α, γ, and ε, as shown in Figs 3 and 4. After autoradiography, the samples were analyzed by cutting out the relevant portion of the gels and counting in a liquid scintillation counter. (---Δ---), α Globin transcript; (---■---), γ globin; and (---○---), ε globin RNA. Error bars indicate the standard error of four independent experiments.

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transcriptional activity in a similarly transfected Chinese hamster ovary cell line was negligible and not affected by incubation of cells with activin A. Previous studies had shown that this nonerythroid cell line possessed receptors specific for activin A molecule (Matthews LS, unpublished observation).

**DISCUSSION**

Activin A is an evolutionarily conserved protein. The β-subunits of activin A have significant homology to transforming growth factor-β (TGF-β) in primary amino acid sequences and distribution of cysteine residues. When the cysteine residues of the β subunit of activin A and TGF-β molecule are aligned with each other, they have 33 amino acid residues in identical positions and have 10 more amino acids with minor conservative changes, thus giving rise to an overall sequence homology of about 30% to 40%. TGF-β has a variety of different biologic activities, depending on its target tissues. Some members of the TGF-β superfamily are closely related to TGF-β. The other members, including inhibin and activin, are more distantly related to TGF-β. This family of TGF-β now includes Mullerian duct inhibiting substance, the decapentaplegic gene complex of Drosophila, and the Vg-1 gene in Xenopus, all of which display differentiation functions. Recently, activin A has been implicated as an important mesoderm-inducing factor in embryogenesis of mice and plays some role during development of Xenopus. It is conceivable that during early development, activin A is an important regulator. Given the evolutionary conservation...
of activin A among different species and the homology between this molecule and other members of the TGF-β superfamily, it is likely that, similar to TGF-β, activin A may have biologic activities on different tissues, playing many roles as hormonal, paracrine, and autocrine regulators.

The chemical and biologic properties of the activin family have recently been reviewed. In these and the accompanying studies, we investigated the effects of activin A on human erythroid cells.

It has been suggested that activin A provides a complex humoral regulatory control over erythropoiesis. Activin A is known to potentiate the number of erythroid colonies in human bone marrow cultures. The potentiation effect of activin A could be attributed to an increase in the proliferative state of BFU-E and CFU-E in the bone marrow cultures. Previously, it was shown that the potentiation effect of activin A on cellular proliferation was mainly observed in erythroid lineage. It is also possible that activin A might drive the differentiation of progeny of these progenitors into hemoglobinized cells, thus rendering the colonies recognizable as erythroid colonies. We have shown in present studies that activin A can induce the accumulation of globin transcripts and stimulate the rate of their transcriptions in K562 cells.

It was found that activin A could increase the expression of globin transcripts for α, γ, and ε globins, while no expression of β globin message was detected. The hybridization protocol used an 80% formamide solution and highly stringent conditions. The absence of cross-hybridization between the β globin probe and other β-like globin mRNAs in this analysis demonstrates the specificity of each probe for its unique mRNA species. This was observed despite considerable homology among β, γ, and ε exon 2 sequences. In addition, the lack of expression of the β globin gene seems to be a special property of the K562 cells, because they were shown to contain intact copies of the adult β globin gene. Previous studies concerning the expression of various globin genes favor an interpretation that K562 cells contain positive trans-acting factors that interact with sequence elements in and around the embryonic and fetal globin genes but lack a comparable trans-acting factor required for β globin gene expression.

The present studies also indicate that most of the increase in the globin transcripts after activin A treatment could be attributed to the stimulation of transcription rate for these globin genes. This observation is in agreement with the finding that activin A stimulated transcriptional activity of a ε globin 5'-flanking sequence in the transfection assay. (The characterization of activin-responsive elements in the globin promoter sequences is under active study and will be presented elsewhere.) This is similar to the observations that hemin seems to extend its effect on globin accumulation in K562 cells at the level of transcriptional regulation. However, hemin does not induce stimulation of transcription from the same ε globin promoter in a similar transfection assay. It should also be noted that hemin did not cause commitment of cells to "terminal differentiation" (defined as limited capacity for further proliferation”), but activin A did induce these cells to become differentiated and associated with limited proliferation. The latter is similar to thymidine and butyrate, which also cause commitment of K562 cells. The accumulation of hemoglobin in the activin-treated K562 cells was shown to represent an approximately 23-fold increase in hemoglobin content. We had estimated that activin A is about twice as effective on a single-cell basis as are chemical inducers in inducing hemoglobin production in responsive K562 cells. Therefore, activin A is capable of exerting a potent differentiation-inducing effect on K562 cells. K562 cells are known to possess approximately 600 high-affinity 125I-activin receptors per cell. In the accompanying report, it was shown that 125I-activin A could be affinity-labeled to the surface binding proteins (45 to 54 Kd) in both purified erythroid progenitors and K562 cells. This affinity labeling of surface binding protein could not be displaced with incubation of excess amounts of erythropoietin. Therefore, the effects of activin A on globin gene expression in K562 cells are most likely mediated through its specific surface receptors, and are independent from the action of erythropoietin.

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Regulation of globin gene expression in human K562 cells by recombinant activin A

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