Active β -Globin Gene Transcription Occurs in Methylated, DNase I-Resistant Chromatin of Nonerythroid Chicken Cells

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We report active, inappropriate transcription of the chicken β^A -globin gene in normal fibroblasts, cultured MSB cells, and brain. We were unable to detect ovalbumin gene transcription in these same tissues. Most of the globin gene transcripts were found to be truncated near the beginning of the gene, suggesting the existence of a premature termination process that is preferentially active under conditions of inappropriate transcription. The inappropriately transcribed β^A -globin gene chromatin remained DNase I resistant and highly methylated. Thus, the DNase I-sensitive conformation of erythrocyte β^A chromatin was correlated not with β^A transcription per se but with β^A expression. Although both transcribed and nontranscribed genes within the globin domain exhibited the same DNase I sensitivity in erythrocyte nuclei, a housekeeping gene active in erythrocytes exhibited a different level of DNase I sensitivity. However, this gene exhibited the same level of DNase I sensitivity in both erythrocytes and a cultured cell line. These observations are consistent with the proposal (G. Blobel, *Proc. Natl. Acad. Sci. USA* 82:8527-8529, 1985) that the DNase I sensitivity of a gene may reflect properties of chromatin related to cotranscriptional and posttranscriptional aspects of mRNA production rather than to transcription per se.

The nature and function of the transcriptionally competent state of chromatin remains an enigma despite much work. Two properties are generally characteristic of competent chromatin: an increased sensitivity to digestion by DNase I and a reduced frequency of cytosine methylation at CpG positions (63). However, the notion of "competent" chromatin is poorly defined. Generally it is taken to mean a region of chromatin that is committed, by virtue of a special conformation, to the present or future transcription of one or more of its resident genes.

Little is known about the nature of the DNase I-sensitive conformation of competent chromatin. The DNase I-sensitive state encompasses both the active genes themselves and the nontranscribed regions of chromatin flanking them. This contrasts with the situation for transcriptionally induced perturbations of chromatin, for which several structural features have been described (3, 14, 18, 22, 40, 58, 67, 79). These perturbations are limited both temporally and spatially to the regions actually undergoing the transcription and are therefore distinct from the all-inclusive DNase I sensitivity of competent chromatin. The large DNase I-sensitive regions characteristic of competent chromatin may include several genes and, in the most completely mapped instances, cover discrete domains that extend uniformly through 10 to 100 kilobase pairs (kb) of chromatin (2, 39, 49). These domains can even include more nontranscribed flanking chromatin than actively transcribed gene chromatin. Moreover, the nontranscribed regions are just as sensitive to DNase I digestion as are the transcribed regions, provided the last-cut approach to the measurement of DNase I sensitivity is used (2, 39, 49, 67, 72).

The last-cut approach to DNase I sensitivity measurement, in the terminology of Jantzen et al. (39), is defined as digestion by DNase I to DNA fragment sizes so small that the DNA no longer hybridizes. In contrast, the first-cut approach measures the rate of restriction fragment disappearance on genomic Southern blots as a function of DNase I digestion. The rate at which these first cuts occur may not be representative of the susceptibility to cutting throughout the region as a whole. In this discussion, and in our experiments, we are interested in the regional, last cut DNase I sensitivity of chromatin.

The function of the chromatin conformation that underlies regional DNase I sensitivity is not clear. The extent of the altered conformation, encompassing large stretches of nontranscribed chromatin together with the transcribed regions, suggests that its function may not be limited to, or even may be different from, the mere mechanical facilitation of chromatin usage by RNA polymerase.

An alternative to the presumption that the DNase I-sensitive conformation of chromatin is designed to make chromatin accessible to the transcription machinery is the possibility that this state of chromatin does not serve a direct transcriptional function but rather is incidental to some other property of chromatin related to gene expression. One such property may be torsional stress (see reference 27 and discussion in reference 73). Another property may be the manner in which the active chromatin is associated with other structures within the nucleus, particularly at the nuclear periphery and in channels leading thereto, so as to provide for appropriate and efficient expression of the designated genes (36, 48). As suggested by Blobel (8) in his gene-gating hypothesis, the three-dimensional arrangement of competent chromatin, and possibly a large share of its DNase I sensitivity, may be related to transport functions associated with the ultimate expression of the transcribed gene. Indeed, de la Peña and Zasloff (19) have shown for the thymidine kinase gene that sequences within the promoter itself are probably involved in transport functions. Thus, the

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chromatin template may be physically involved in all steps of gene expression up to the exit of the RNA from the nucleus.

Hypomethylation of competent chromatin, like DNase I sensitivity, is poorly understood. Indeed, it is not yet possible to describe an all-encompassing relationship between transcription and the level of DNA methylation (7, 11, 28, 63, 69). In general, however, hypomethylation is correlated with gene activity, a correlation that has been particularly well established with respect to the normal developmental regulation of the β -globin gene family in several organisms (10, 28, 30, 31, 52, 54, 66, 70). Consistent with a role for methylation in gene commitment, Keshet et al. (43) have shown that transfected unmethylated DNA is assembled into an active-like chromatin structure, whereas transfected methylated DNA is assembled into inactive chromatin. Similarly, certain genes, in cell lines whose chromatin has been stably demethylated by treatment with 5-aza-2'-deoxycytidine, exhibit increased DNase I sensitivity of their chromatin (56). Moreover, transfection experiments have established that in many cases methylation of DNA *in vitro* interferes with subsequent expression *in vivo*. Notably, Buschhausen et al. (9) have reported that this inhibition occurs only after the formation of chromatin. These effects of methylation are regional in nature (43, 60), although they are not propagated through the enormous distances characteristic of regional DNase I sensitivity.

Thus, methylation may assist in the regulation of transcription via modifications in chromatin structure. Of course, direct effects via modification of factor-binding sites presumably also occur (4, 44) independently of the postulated regional effects. However, it is also possible that the overall methylation status of chromatin is involved in transcriptional regulation only indirectly. Its primary role may be related principally to aspects of chromatin organization involved in subsequent steps of gene expression, as suggested above for DNase I sensitivity.

One way to explore the possibility that DNase I sensitivity and methylation may be related to more global aspects of gene expression, beyond the primary level of transcription itself, is to examine the relationships among these parameters under biological circumstances in which expression of a transcribed gene lies clearly outside the repertoire of functions available to the cell type involved. In such a situation, the inappropriate transcription that occurs may be uncoupled from the putative nuclear rearrangements of chromatin designed to facilitate ultimate expression (see above).

Humphries et al. (34) have described a clear case of inappropriate transcription in the mouse. By means of direct hybridization to nuclear RNA (i.e., without amplification via the polymerase chain reaction), they found globin RNA at significant levels in nuclei from several nonerythroid cell lines and tissues, including brain. Here we confirm their finding for various chicken cell types and show that the inappropriate transcription is not accompanied either by hypomethylation of the DNA or by generalized DNase I sensitivity of the chromatin.

MATERIALS AND METHODS

Isolation of erythroid cells. Fertilized eggs were obtained from Chino Valley Ranchers, Chino, Calif., and stored at 10 to 15°C for at least 1 day before incubation in a Humidaire (New Madison, Ohio) model 50 incubator at 37.5°C (dry bulb). Five-day embryos were bled by heart puncture, followed by filtration through several layers of cheesecloth to remove heart fragments. Six-day embryos were bled by

puncturing the veins on the vitellin membrane, and thirteen-day eggs were bled by cutting the main blood vessels over the yolk sac. Blood was collected into cold phosphate-buffered saline (PBS; 140 mM NaCl, 1.7 mM KCl, 1.5 mM KH_2PO_4 , 8 mM Na_2HPO_4). Cells were then washed twice and resuspended in PBS. Cell number was determined by use of a hemacytometer. Six-day cells to be fractionated into primitive (light) and definitive (dense) components were resuspended in 4.5 ml of PBS, mixed with Percoll (Pharmacia, Uppsala, Sweden) and centrifuged for 30 min.

Separation of cells in Percoll gradients. Between 10^8 and 10^9 cells in PBS were added to 30 ml of Percoll made isotonic by addition of $30\times$ PBS (1 volume of $30\times$ PBS plus 29 volumes of Percoll at 1.130 g/ml to give a final density of 1.132 g/ml). The cells and Percoll were mixed by inversion. The gradients were self-generated by centrifugation at $27,000 \times g$ and then fractionated by bottom puncture. The cells were then freed of Percoll by three washes in cold PBS.

Isolation of brain cells. The brains of three to five decapitated 19- or 20-day chicken embryos were washed with several changes of ice-cold PBS to remove contaminating erythrocytes. The washed brains were either used directly for isolation of nuclei or, when specified in the text, first dispersed with trypsin and then purified on a Percoll gradient before isolation of the nuclei. In preparation for loading on a Percoll gradient, the brains were passed rapidly through a 1-ml syringe, and the resulting fragments were allowed to settle. The supernatant was discarded, and 10 ml of a warm 0.25% trypsin (GIBCO Laboratories, Grand Island, N.Y.) solution was added. After a few seconds of vigorous pipetting, all remaining large fragments were allowed to settle, and the supernatant containing the dispersed cells was withdrawn and treated with 0.1 ml of a 25- $\mu\text{g}/\text{ml}$ concentration of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.). The fragments were cycled through two more treatments with trypsin in the same fashion, and all of the resulting supernatants were then pooled and centrifuged at $850 \times g$ for 3 min at 4°C. The pelleted cells were suspended in 4 ml of PBS, mixed with 30 ml of isotonic Percoll, and centrifuged for 15 min.

Cell culture. Primary cultures of chicken embryo fibroblasts were established and passaged by the method of Hunter (35). Cells were used during the second, third, or fourth passage. The MSB cells were obtained from Vaughn Jackson (38). This cell line was originally established by Akiyama and Kato (1) from a splenic lymphoma from a chick with Marek's disease. The cells were grown on dishes in Dulbecco minimal essential medium containing 10% newborn calf serum or in suspension as described previously (38) but with a 2:1 ratio of Dulbecco minimal essential medium to RPMI 1640.

Run-on transcription. Nuclei were isolated as described by Landes and Martinson (45) and Fodor and Doty (25) by homogenization in Triton X-100 and sucrose. The nuclei were centrifuged through a 1.5 M sucrose pad at $5,000 \times g$ and then suspended in a buffer containing glycerol and kept at -70°C until used. Run-on transcription was carried out as previously described (45) at 25°C for 15 min in 150 mM KCl in the presence of $[\alpha\text{-}^{32}\text{P}]\text{CTP}$ (5,000 Ci/mmol). Transcript isolation was by method II of Landes and Martinson (45).

Isolation and end labeling of RNA. Whole cells or nuclei were lysed by addition of 2 volumes of 6 M guanidinium isothiocyanate (containing 0.75% Sarkosyl [CIBA-GEIGY Corp., Summit, N.J.], 37.5 mM sodium citrate, 0.15 M β -mercaptoethanol, and 0.2% antifoam, adjusted to pH 7.0 and filtered through 0.22- μm -pore-size nitrocellulose [Milli-

pore Corp., Bedford, Mass.) and immediately homogenized with a motorized Teflon pestle until viscosity was greatly reduced (16). The homogenate (4 to 8 ml) was layered onto a 2 to 4 ml CsCl pad (5.7 M CsCl, 25 mM sodium acetate [pH 5.0]) and then centrifuged at 40,000 rpm in a Ti-75 rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 15 to 20 h at 20°C. The RNA pellet was suspended in 10 mM Tris-1 mM EDTA (pH 7.8), extracted twice with CHCl_3 , and then ethanol precipitated. The RNA was hydrolyzed on ice (30 min in 0.2 N NaOH-0.1% sodium dodecyl sulfate), neutralized, ethanol precipitated, end labeled with T4 polynucleotide kinase (1 mCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ per μg of RNA), and desalted (61).

Cloned DNAs. The genomic globin clones used are described by Villeponteau et al. (71). The ovalbumin cDNA clone is pOV230 as described by McReynolds et al. (55). For actin and tubulin probes, we used the DNA clones for β -actin (pA1) and β -tubulin (pT2) constructed by Cleveland et al. (17). The glyceraldehyde-3-phosphate dehydrogenase (GD) probe (pG2GAD1.5) was kindly provided by Ming-Jer Tsai and contains 843 base pairs (bp) from the 5' end of the gene plus 668 bp of 5'-flanking DNA. This clone is a derivative of one of the genomic clones described in Alevy et al. (2).

Hybridization. The methods for Southern blotting and hybridization were essentially those of Landes et al. (46) for Fig. 1 to 5 and Villeponteau et al. (72) for Fig. 6 and 8. Basically, hybridization was carried out in 50% formamide at 43°C, and the final posthybridization wash was at 65°C in 15 mM NaCl-1.5 mM sodium citrate. Plasmid DNA for dot blotting was depurinated in 0.2 M sodium acetate (pH 4) at 55°C for 15 min before use. All DNA was alkali denatured, neutralized, and then either brought to approximately 1.5 M NaCl-0.15 M sodium citrate, applied to nitrocellulose, and baked (Fig. 2 and 4) or brought to approximately 3 M NaCl-0.3 M sodium citrate, applied to Nytran, and UV cross-linked (Fig. 8).

RESULTS

The β^A -globin gene is actively transcribed in brain. Nuclei were isolated from carefully washed brain tissue of 19-day chicken embryos and subjected to run-on transcription in vitro. The labeled transcripts were hybridized to a Southern blot of restriction enzyme-digested DNA spanning the chicken β -like globin gene region (46, 71). The resulting transcript autoradiogram showed the usual hybridization of RNA to repeat sequences in the DNA. However, two lanes (Fig. 1) containing the restriction digests of plasmid clones spanning the β^A -globin gene were remarkable. These lanes revealed active transcription of the β^A -globin gene in the brain. All DNA in and immediately surrounding the β^A -globin gene is unique in the genome (71), and the closest repeated sequence lies more than 1 kb to the 3' side of the structural gene (within *Hpa*II fragment 16.5). Therefore, the transcripts come from the β^A -globin gene itself and not from elsewhere in the genome. We have determined that this transcription is α -amanitin sensitive (0.2 $\mu\text{g}/\text{ml}$) and therefore mediated by RNA polymerase II (data not shown).

Interestingly, the brain transcript autoradiogram from this experiment was, except for the two lanes of Fig. 1, almost indistinguishable from a previously published repeat-sequence blot of the globin region (Fig. 2 of reference 71). For the repeat-sequence blot, nick-translated chicken genomic DNA, rather than run-on RNA, was hybridized to a Southern blot of restriction enzyme-digested clones in order to

FIG. 1. Transcription of the β^A -globin gene in brain cell nuclei in vitro. Nuclei were subjected to run-on transcription in vitro for 15 min. The labeled transcripts were hybridized to a Southern blot of clone pCBG13 digested with *Hha*I and clone pCBG15 digested with *Hpa*II. Map positions of the clones and their restriction patterns (C) are from Villeponteau et al. (71), with slight revisions based in part on sequence data from Dolan et al. (21). (A) Autoradiogram of the Southern blot; (B) a histogram derived from it. Dotted segments in the base line of the histogram indicate small regions for which we have no data. The Southern blot was quantitated as previously described (46) except that the actual number of counts hybridized was determined directly by excising and counting representative bands from the Southern blot rather than by carrying out a separate hybridization.

detect DNA repeat families in the region. Thus, most of the repeat sequence families that are represented within the β -like globin gene region are also represented within transcription units that are active in the brain.

The transcription of the β^A -globin gene in brain nuclei is surprising not only for its mere existence but also for its level of activity. The autoradiogram in Fig. 1A was scanned, and the extent of hybridization was quantitated (Fig. 1B). The results revealed that the level of transcription was more than 10% of that for active (9-day embryonic) definitive erythrocytes themselves (50). Moreover, within the resolution of these blots, the boundaries of the transcription unit were the same in brain as in erythrocytes (71). However, unlike in erythrocytes, the pattern of run-on transcription across the β^A -globin gene in the brain nuclei was unusual in that there

was a strong signal from the 5' end of the transcription unit (fragment 13.5 in Fig. 1) but a weaker signal from the middle of the gene. This finding indicates either that the polymerase density is greater near the beginning of the gene as a result of pausing or that many transcripts are aborted early in the transcription unit. The strong signal over fragment 16.5 is presumably a consequence of the repeat sequence in that segment.

The different pattern of β^A -globin gene transcription in the brain versus that in erythrocytes argues against the possibility that contaminating erythrocyte nuclei were actually the source of the β^A -globin transcripts in our brain preparations. Nevertheless, to eliminate erythrocyte contamination as well as to obviate any possibility of being misled by some artifact of the run-on transcription procedure, we repeated the experiment with two important modifications. First, we freed the brain cells entirely of erythrocyte contamination by banding in Percoll, and then we isolated and examined directly the brain cell steady-state nuclear RNA. The analysis of steady-state RNA also introduced an additional criterion by which to judge the cellular source of the transcripts. Erythrocytes, but not brain cells, carry a large load of stable globin mRNA in their cytoplasm which inevitably contributes substantially to the RNA content of the nuclei as prepared by our rapid procedure (e.g., Fig. 2A). Contamination of the brain nuclei with erythrocyte nuclei would therefore be indicated by the presence of globin mRNA, which of course is recognizable by the presence of exon and the absence of intron sequences. Therefore, any preparation of brain nuclei that yields nuclear RNA containing mostly globin exonic sequences would be deemed to be contaminated with erythrocyte nuclei. Conversely, if globin transcripts containing equivalent amounts of intron and exon are found in the brain RNA, it would be clear that these could not have arisen from erythrocyte contamination, and we would therefore regard them as being manifestly of brain origin.

To isolate clean brain cells, we began by dispersing the washed brain tissue with trypsin until the cells were free of aggregates. Then we banded the suspended brain cells in a Percoll gradient to remove any contaminating erythrocytes. The brain cells form a white band at the very top of the gradient, whereas the few erythrocytes present form a narrow band at the very bottom. Nuclei were then isolated, lysed in 6 M guanidinium isothiocyanate, and centrifuged over a pad of CsCl by the method of Chirgwin et al. (16) to pellet the RNA. The RNA was then fragmented by using alkali, end labeled with ^{32}P , and hybridized to a Southern blot similar to that of Fig. 1.

The hybridization pattern for the nuclear RNA from brain is shown in lanes 1 and 2 of Fig. 2A. There was hybridization to all three regions of the β^A -globin gene, i.e., fragments 13.5, 14.1, and 14.8' (see Fig. 1C; 14.8' is essentially the same as 14.8 [see legend to Fig. 2]). Moreover, the relatively strong hybridization to fragment 14.1, which was mostly intron, confirmed that the globin sequences were mostly nuclear in origin and that they came from brain. Nuclear RNA as prepared by us from erythrocytes (Fig. 2A, lanes 3 and 4) was indistinguishable from total (i.e., cytoplasmic) erythrocyte RNA (lanes 5 and 6) and hybridized mostly to fragments 13.5 and 14.8', which contained the most exon. Therefore, the RNA in lane 1 of Fig. 2A, which hybridized well to fragment 14.1 but less well to fragment 13.5, was not of contaminating erythrocyte origin but was genuine brain RNA. The fact that brain nuclei gave an excess of hybridization to fragment 13.5 for run-on transcripts but not for

FIG. 2. Transcription of the β^A -globin gene in brain cells in vivo. Nuclear RNA was isolated from Percoll-banded cells of 20-day embryo brains or from 15-day embryo erythrocytes. Total RNA was isolated from 16-day erythrocytes. The RNA was end labeled and then hybridized either to Southern blots of clones pCBG13 and -15 digested with *Hha*I (A) or to a dot blot of these same two clones plus the cDNA clones for β -tubulin and ovalbumin (B). In panel A, the mobility of fragment 14.8' is less than that of 14.8 in Fig. 1 because of the difference in restriction enzyme used to cleave clone pCBG15. Fragment 14.8' contains a little more globin DNA on the right but much less vector DNA on the left (see reference 71 for details). The undesigned fragment visible high in lane 2 for brain but not for erythrocyte RNA is from the right-hand end of pCBG15 and contains a genomically repeated sequence from the 3'-flanking region of the β^A gene.

steady-state RNA is consistent with the possibility that in brain, the polymerases accumulate transiently in the early part of the gene because of pausing but then continue downstream to complete transcription of the gene. Alternatively, if premature termination of transcription was occurring in segment 13.5, then the aborted transcripts clearly were less stable than complete transcripts in the brain. Such results have been reported for attenuated transcripts of human immunodeficiency virus type 1 in COS7 cells (41) and of *c-myc* in HL60 cells (23).

For comparison, we also measured the nuclear steady-state levels of tubulin and ovalbumin RNA in brain. The brain nuclear globin transcripts were at least 10% as abundant at tubulin transcripts (Fig. 2B), which confirmed the conclusion from the data of Fig. 1 that the globin gene is very active in brain and reinforced the conclusion that the amount of globin gene transcription in brain far exceeded that which could be accounted for by the undetectable levels of eryth-

FIG. 3. Transcription of the β^A -globin gene in fibroblast nuclei. Transcription, hybridization, and quantitation were as for Fig. 1.

rocyte contamination present. Conversely, there were no detectable ovalbumin transcripts in the brain RNA. This finding suggested that transcription of the globin gene in brain reflects an unusual property inherent to the globin gene itself rather than some general characteristic of brain transcription involving the promiscuous activity of many genes. In particular, this phenomenon is not related to the "accidental" transcription of inactive genes that has recently been reported (13, 65). Unlike the inappropriate transcription described here, accidental transcription is so rare that it can be detected only after amplification of the RNA by the polymerase chain reaction.

The β^A -globin gene is actively transcribed also in fibroblasts and MSB cells. To confirm that it is the globin gene itself rather than the brain in general which is unusual, we also assayed for globin gene transcription in cultured fibroblasts and in an established lymphoid cell line. Using cultured cells provides the additional advantage that any possibility of erythrocyte contamination is eliminated.

Like brain, fibroblasts exhibited active β^A -globin gene transcription in nuclear run-on experiments but with an even more extreme 5' bias (Fig. 3). Also as for brain, the fibroblast nuclei produced transcripts homologous to almost all of the repeat families represented in the globin region (not shown), including the repeat in fragment 16.5 (Fig. 3).

MSB cells also engaged in active β^A -globin gene transcription (Fig. 4). Nuclear RNA was end labeled and hybridized to a slot blot of various cloned DNAs in a manner similar to the brain experiment of Fig. 2B. As for brain, the concentration of globin RNA (pCBG13 and its subclone 13.5) was substantial even compared with that of actin and tubulin, but there was no detectable signal for ovalbumin RNA (Fig. 4). Like fibroblasts, the MSB cells exhibited an almost exclu-

sive bias toward the 5' end of the gene (i.e., the RNA was homologous only to clones 13 and 13.5, not to clones 15 and 14.1; see Fig. 1C). We note that if, as may be the case for brain, truncated transcripts are less stable than full-length transcripts in MSB cells, then this experiment underestimates the amount of globin transcription by severalfold.

As an independent and more rigorous test of the authenticity of the partial globin transcripts detected by slot blotting (Fig. 4A), we carried out a mung bean nuclease protection assay of the MSB cell nuclear RNA. The MSB cell nuclear RNA efficiently protected the globin probe (Fig. 4B, lane 4), yielding three or four principal digestion products (see legend to Fig. 4B). In contrast, yeast RNA (lane 6) did not protect the globin probe at all. Erythrocyte RNA (lane 5), of course, protected the globin probe very well and yielded a major digestion product whose length was consistent with the known principal start point of transcription for the β^A -globin gene in erythrocytes (21). However, comparison of the MSB and erythrocyte lanes reveals that although the MSB cell nuclear RNA contained genuine globin transcripts, the 5' ends of these transcripts lay upstream of the tissue-specific 5' ends of the β^A -globin mRNA from erythrocytes.

Two details in Fig. 4B require further comment. First, in lane 3, for which the probe was not treated with nuclease, the bottom band has been observed in varying proportions in different gels and presumably represents a small proportion of the probe that runs in double-stranded form. Bands in lanes 4 and 5 at a similar position may have the same origin, but this seems unlikely. The top band in lane 3 is at the normal position for full-length probe (153 nucleotides) running in single-stranded form. The other item requiring comment is the top band in lane 4. This band, at the position of full-length probe, suggests the presence of RNAs with 5' ends lying further upstream than the end of the probe. The lack of such a band in lanes 5 and 6 shows that the nuclease digestion was complete under our conditions.

Inappropriate transcription of the β^A -globin gene is characterized by 5' truncation even in certain erythroid cells. Fibroblasts, brain, and MSB cells were not the only sources of inappropriate β^A -globin gene transcription. Primitive erythroid cells, specialized for the expression of the embryonic hemoglobins and in which no adult hemoglobin synthesis can be detected even by sensitive immunofluorescent techniques (12), also engaged in inappropriate transcription of the β^A -globin gene (Fig. 5). As for the nonerythroid cells, this transcription was prematurely diminished near the beginning of the gene (Fig. 5B and C). In contrast, definitive cells, specialized for adult hemoglobin synthesis, displayed much more consistent transcription levels across the gene (Fig. 5D). The contrast between the definitive cells (Fig. 5D) and the primitive cells (Fig. 5C) is particularly noteworthy because these two populations of cells were partially purified from the same 6-day embryos, whose blood was fractionated by Percoll density gradient centrifugation.

The variability in transcription of segment 12.8 is probably not significant to these results. This segment contains a short RNA polymerase II transcription unit of orientation opposite that of the globin genes which varies considerably in activity from experiment to experiment (62; T. A. Pribyl, unpublished).

The β^A -globin gene in brain is highly methylated. Since the entire β^A -globin gene appears to be transcribed in brain (Fig. 1 and 2), we decided to investigate its methylation status at the *Hpa*II and *Hha*I sites in its vicinity (Fig. 6). Methylation is detected as lack of cutting at known restriction sites, since

FIG. 4. Transcription of the β^A -globin gene in MSB cells in vivo. (A) Nuclear RNA from MSB cells or from 16-day erythrocytes (Eryth) was end labeled and then hybridized to a slot blot containing the various cloned DNAs indicated. Nuclei were prepared as for run-on transcription. Each hybridization was to duplicate slots of cDNA clones for actin, tubulin, and ovalbumin (Oval) as well as to the genomic globin clones and subclones indicated (see Fig. 1C for map positions). As discussed in the text concerning Fig. 2, the erythrocyte nuclear RNA was contaminated with significant amounts of cytoplasmic mRNA and therefore hybridized most strongly to exon-containing probes. (B) The mung bean nuclease protection assay was carried out as described by Dolan et al. (21). Lanes: 1, *HinfI* digest of pBR322; 2, *DdeI* parent fragment to the *SmaI-DdeI* hybridization probe; 3, the 153-nucleotide 5'- 32 P singly end-labeled *SmaI-DdeI* hybridization probe which spans the β^A -globin mRNA cap site (the minor band is presumably some renatured probe migrating in double-stranded form); 4 to 6, 200 μ g of RNA hybridized to 70 ng of probe; 4, MSB cell nuclear RNA prepared from nuclei isolated as described by Villeponteau et al. (72); 5, cytoplasmic RNA from 15-day embryonic erythrocytes prepared according to Dolan et al. (21); 6, yeast total cellular RNA (Sigma). Eryth, Erythrocytes.

these enzymes are inhibited by cytosine methylation at the CpG positions of their recognition sequences.

First, we confirmed and extended (Fig. 6, erythrocyte [Eryth] lanes) the results of others who showed that the degree of methylation of the chicken β -globin genes reflects the transcriptional status of the genes in erythroid cells (30, 52). We used the isoschizomer pair *HpaII* and *MspI* to digest brain or erythrocyte DNA for the preparation of genomic Southern blots. *MspI* serves as a control for restriction site polymorphism because it is unaffected by methylation at CpG. For example, the 0.66-kb bands detected by an ϵ -gene

intron probe in lanes 1 and 2 of Fig. 6 revealed a known *MspI* restriction site polymorphism near the 3' end of the ϵ -gene large intron. DNA that lacked a site at this position gave the 3.1-kb band instead (Fig. 7). The bands at 1.22 and 3.7 kb are probably explained by polymorphism at a second *MspI* site at the other end of the intron (Fig. 7), although the presence of m CpC in some of the sites may also be the explanation (68).

For DNA from 5-day cells, in which the ϵ gene is expressed, *HpaII* cutting near the ϵ gene was efficient and methylation was therefore low (Fig. 6, lane 3). Conversely,

FIG. 5. Appropriate and inappropriate transcription of the β^A -globin gene in erythroid cells. Primitive and definitive cells, fractionated from 6-day embryo blood, and primitive cells from 5-day embryos were prepared. Isolation of nuclei, transcription, hybridization, and quantitation were as described for Fig. 1. The transcription signals in the histograms were normalized to that of fragment 13.5 to facilitate comparison, among panels, of transcription ratios along the gene. To evaluate the autoradiograms directly, compare the signals for bands 13.5 and 15.4 in each set.

for DNA from 13-day cells, in which the ϵ gene is not expressed, *Hpa*II cutting was inefficient surrounding the ϵ gene and methylation was therefore high (lane 4). For both *Hpa*II (Fig. 6, lanes 8 and 9) and *Hha*I sites (lanes 11 and 12), the reciprocal situation existed for the β^A gene, which is expressed at 13 days but not at 5. These results confirm the correlation between expression and hypomethylation of genes. The results also illustrate that the relationship between methylation and DNase I sensitivity is not a simple one because both the ϵ and the β^A genes are uniformly DNase I sensitive at 12 days (72, 78). Thus, DNase I sensitivity is correlated with the commitment of domains, whereas hypomethylation is correlated with the expression of individual genes.

In brain, the β^A gene was methylated even more extensively than in the nonexpressing 5-day erythrocytes (Fig. 6, compare lanes 10 and 13 with lanes 9 and 12). For *Hpa*II, the principal band was at 4.4 kb (lane 10), and for *Hha*I the principal signal detected was a smear above 9 kb (bracketed region of lane 13). All of these methylation results are summarized in Fig. 7.

The β^A -globin gene of nonerythroid cells does not exhibit the DNase I sensitivity of active chromatin. The results of Fig. 1 to 4 demonstrated, either by run-on transcription of isolated nuclei or by direct isolation and assay of nuclear RNA, that active transcription of the β^A -globin gene occurs in brain, fibroblasts, and MSB cells. However, it is well documented that the chicken β -globin gene domain does not exhibit the preferential DNase I sensitivity of active chromatin in either brain cells (72, 76, 78) or fibroblasts (76, 77). Here we confirm a similar result for MSB cells.

Figure 8 shows the relative DNase I sensitivity of the β^A -globin gene in MSB cells compared with that of erythrocytes. DNA from MSB or erythrocyte nuclei that had been digested briefly with DNase I was dotted on Nytran and hybridized to the following probes: (i) a globin probe that covers the 5' third of the β^A gene, (ii) a probe for the GD gene that is active in all cells, and (iii) an ovalbumin probe to whose hybridization the results of the other two probes were normalized. The results showed that whereas the GD gene exhibited a similar, moderate sensitivity to DNase I in both cell types, the β^A -globin gene was very sensitive to DNase I in erythrocyte nuclei but not in MSB cell nuclei. Thus, despite the fact that transcription occurs at the globin gene in both cell types, its chromatin conformation differs dramatically.

DISCUSSION

Inappropriate transcription of the β^A -globin gene. The presence of a substantial number of globin transcripts in brain cells (Fig. 1 and 2) was first reported for the mouse by Humphries et al. (34), who presented strong arguments against an artifactual explanation. We have carried out an even more rigorous set of controls to rule out completely any explanation related to contamination by erythrocytes. The results (Fig. 2) show that brain cells, freed of erythrocyte contamination by banding in a Percoll gradient, still contain β^A -globin RNA in their nuclei. Moreover, this globin RNA possesses intron sequences, thus ruling out, by an independent criterion, any explanation involving contamination by erythrocyte cytoplasmic RNA.

Transcription of the β^A -globin gene in brain cells does not appear to have a trivial explanation. In particular, cross-hybridization to distantly related transcripts from other regions of the genome can be ruled out on several counts.

FIG. 6. *MspI*, *HpaII*, and *HhaI* digestion of the ϵ - and β^A -globin gene regions in brain and erythrocyte DNA. DNA (3.5 μ g) from 5- or 13-day erythrocytes (Eryth) or from 13-day brain (Br) was digested for 4 h at 37°C with 36 U of the indicated enzyme. The DNA was electrophoresed on 1.2% agarose gels, Southern blotted, and hybridized to either an ϵ - or β^A -intron probe.

FIG. 7. Summary of the methylation status at *HpaII* and *HhaI* sites surrounding the β^A -globin gene in brain (Br) DNA and in 5-day (5) and 13-day (13) erythrocyte DNA. *HpaII* sites are represented as vertical lines above and *HhaI* sites are represented as vertical lines below the map (based on data in references 20, 21, 64, and 71). Dotted lines represent sites that are polymorphic. The fragments diagrammed below the map illustrate the fragment length heterogeneity that results from some of these polymorphisms. Fragment lengths are given in kilobase pairs. The methylation summary is intended to be qualitative, not quantitative. An open circle indicates that a site is mostly unmethylated; two closed circles indicate that a site is mostly methylated; one closed circle indicates partial methylation. The probes used for hybridization are shown as shaded bars within the introns of β^A and ϵ and are unique-sequence 519-bp *HaeIII* and 286-bp *HaeIII-EcoRI* fragments, respectively.

FIG. 8. DNase I sensitivity of β^A -globin gene chromatin in MSB cells and erythrocytes (Eryth). DNase I sensitivity assays were carried out essentially as described previously (72). The digestion times were 10 min for MSB cells and 3 min for erythrocytes at the DNase I concentrations indicated. The differing times were chosen to yield similar extents of digestion of the two types of nuclei for equivalent DNase I concentrations. The DNA isolated from the digested nuclei was hybridized by slot blot (1.5 μg of DNA per slot) to three probe sequences labeled with ^{32}P by the random-priming procedure (24). The probes were GD (a glyceraldehyde-3-phosphate dehydrogenase probe), 13.6 (a 738-bp *HinfI* fragment encompassing the first two exons of the β^A -globin gene plus 200 bp of 5'-flanking DNA), and pOV230 (an ovalbumin cDNA probe used to normalize the hybridization results of GD and 13.6 as previously described [72]).

First, transcription from the entire length of the gene is observed (Fig. 1 and 2). Second, except for some homology with the other globin gene exons, the β^A sequence is unique in the genome, as revealed by genomic Southern blots probed either with the complete gene sequence or with just the 5' end (fragment 13.5; data not shown). Third, the effective stringency of the aqueous washing step used for the RNA-DNA blots shown here is greater than that for a typical DNA-DNA Southern blot (data not shown), thus discriminating further against any distantly related sequences that may not show up on a genomic Southern blot. Finally, the β^A gene is unique among the globin genes in exhibiting the promiscuous transcription described here. It is unlikely that any family of sequences in the genome, too distantly related to be picked up by a β^A probe on a genomic Southern blot yet abundant enough to show up on a transcript autoradiogram, would be able to discriminate during hybridization among the closely related members of the β -globin family.

Transcription of the β^A gene in brain is α -amanitin sensitive; therefore, it cannot be the result of promiscuous transcription by RNA polymerase III. However, we have not yet determined where transcription initiates in brain cells. The small amount of RNA homologous to sequences upstream of the β^A promoter (Fig. 1, fragment 12.8) may be due either to upstream initiation of transcription as in MSB cells (Fig. 4B) or to a divergently oriented transcription unit that is active in that region in erythrocytes (62).

Humphries et al. (34) reported the presence of globin RNA also in various established mouse cell lines. However, although erythrocyte contamination is precluded in such experiments, the fact that the transcriptional derangements which accompany transformation of cells in culture can include activation of globin genes (32) complicates the interpretation. Therefore, the presence, even in normal fibroblasts, of transcription of a portion of the β^A -globin gene (Fig. 3) is significant. However, the stability of the RNA

produced is apparently quite low, since no globin sequences can be detected in steady-state RNA from normal fibroblasts (32).

Truncation or attenuation in the β^A -globin gene. An excess of polymerases in the 5' half of the chicken β^A gene was first noticed in the erythrocytes of adult chickens by Gariglio et al. (26) and also appears irregularly in the definitive erythrocytes of embryos (71). Gariglio et al. (26) suggested that impaired elongation in the early part of the gene accounted for the accumulation of polymerases and may be part of the mechanism by which transcription is suppressed in the aging erythrocyte. A 5' bias correlated with the lack of β^A expression is also present in the nuclei of primitive erythrocytes, which do not express the adult globins (Fig. 5). However, the 5' bias does not always appear in these cells (46), and a similarly variable 5' bias has also been reported for the mouse β^{maj} -globin gene in Friend cells (59).

In contrast to the variability of the 5' bias for the β^A gene in erythroid cells, its occurrence is much more consistent in nonerythroid cells. The most extreme examples of 5' bias occur in chicken embryo fibroblasts (Fig. 3) and MSB cells (Fig. 4). The effect is not as extreme for brain (Fig. 1). For both fibroblasts and brain cells, where the 5' bias is seen only in run-on transcription but not in the isolated RNA, the results can be explained either by premature termination of transcription (assuming that the truncated transcripts are unstable) or by stably paused polymerases on the template that then run on during the in vitro assay. For MSB cells, in contrast, the only simple interpretation is premature termination of transcription because the 5'-truncated transcripts are readily detectable in the nuclear RNA (Fig. 4).

The occurrence of pausing or premature termination of transcription in the 5'-proximal portions of eucaryotic transcription units has been described for a number of genes in addition to the chicken β^A -globin gene studied here and the mouse β^{maj} -globin gene of Friend erythroleukemia cells mentioned above. For example, Gilmour and Lis (29) have described protein-DNA cross-linking experiments that show an accumulation of polymerases near the 5' ends of several *Drosophila* genes, and Chinsky et al. (15) have shown, by run-on analysis, that the mouse adenosine deaminase gene exhibits 5' truncation of transcription in tissues in which expression of the gene is low. However, the most fully characterized examples are found among viral transcription units (33, 37, 41, 51, 57, 74), cellular oncogenes (5, 6), and the immunoglobulin μ - δ transcription unit (47, 53, 80).

Curiously, most of these examples are of systems that are subject to sophisticated regulation in vivo. However, for the globin genes, regulation is relatively straightforward. This suggests that regulated elongation (pausing or attenuation) near the beginnings of genes may be a common eucaryotic transcriptional regulatory mechanism, not an unusual one reserved for exotic patterns of regulation.

DNase I sensitivity, methylation, and gene expression. We have shown that all or part of the chicken β^A -globin gene is transcribed inappropriately in several tissues in which it is presumably never expressed. However, this transcription is not accompanied by either hypomethylation of the DNA or a significant degree of DNase I sensitivity in the chromatin. Not surprisingly, DNase I sensitivity, long regarded as the hallmark of active chromatin, has rarely been studied under circumstances in which the associated transcription could be regarded as trivial. The results presented here, which constitute just such a study, suggest that DNase I sensitivity is, in fact, not correlated with all types of transcription but only with appropriate transcription, i.e., transcription in the

context of potential, biologically appropriate expression. Thus, DNase I sensitivity at the level of domains, and possibly also DNA methylation at the level of individual genes (see Results), may be related more consistently to the ultimate, and appropriate, expression of genes rather than simply to their transcription. Accordingly, in brain, where the β^A -globin gene will never be expressed, it is methylated and DNase I resistant despite active transcription.

Kelley et al. (42) have come to a strikingly similar conclusion on the basis of a study of an unrelated system. They found that vigorous and prolonged but "precocious" κ immunoglobulin gene transcription, which can be induced by lipopolysaccharide treatment of cells in a pre-B line, is not accompanied by hypomethylation. In contrast, the constitutive transcription that occurs, appropriately, in the more differentiated cells of a B-cell line is accompanied by hypomethylation of the DNA. A similar failure to hypomethylate in conjunction with induced transcription has in fact also been reported for the chicken β -globin genes in lines of transformed erythroid precursor cells induced in culture (75).

The failure of inappropriately transcribed globin chromatin in nonexpressing tissues to exhibit DNase I sensitivity demonstrates that the DNase I-sensitive state is not a mechanistic prerequisite for transcription. Rather, this observation is consistent with the suggestion that the DNase I sensitivity of appropriately transcribed genes reflects a conformational state of chromatin associated with aspects of reorganization designed to facilitate RNA processing and transport (see introductory remarks). Of course, a concurrent role in the regulation of transcription itself is not ruled out, but the data suggest that the DNase I-sensitive state is involved in, or is a consequence of, more far-reaching aspects of gene regulation than just transcription per se.

Of interest in this connection is the observation (Fig. 8) that in erythrocytes, the β^A -globin gene is much more sensitive to DNase I than is the GD gene, a housekeeping gene. In contrast, in MSB cells the β^A -globin gene is much less sensitive than the GD gene to DNase I, yet the GD gene exhibits a similar, intermediate degree of DNase I sensitivity in both erythrocytes and MSB cells. These high (globin in erythrocytes), medium (GD in MSB cells), and low (globin in MSB cells) levels of DNase I sensitivity happen to correlate, respectively, with high, medium, and low levels of gene expression. But we know that even among appropriately transcribed genes, the degree of DNase I sensitivity does not correlate simply with the rate of transcription. In 12-day chicken embryo erythrocyte nuclei, the β^A gene is the most active gene in the globin domain, whereas the ϵ gene is hardly active at all (46), yet the DNase I sensitivities of these two genes are the same (72, 78). It therefore appears likely, in accord with the suggestions of Blobel (8) and Hutchison and Weintraub (36), that the intermediate and high levels of DNase I sensitivity observed in erythrocytes for the GD and globin genes, respectively (Fig. 8), correlate with those aspects of cotranscriptional and posttranscriptional mRNA production that differ for these two genes.

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LITERATURE CITED

1. Akiyama, Y., and S. Kato. 1974. Two cell lines from lymphomas of Marek's disease. *Biken J.* 17:105-116.

2. Alevy, M. C., M.-J. Tsai, and B. W. O'Malley. 1984. DNase I sensitive domain of the gene coding for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. *Biochemistry* 23: 2309-2314.
3. Allegra, P., R. Sterner, D. F. Clayton, and V. G. Allfrey. 1987. Affinity chromatographic purification of nucleosomes containing transcriptionally active DNA sequences. *J. Mol. Biol.* 196: 379-388.
4. Becker, P. B., S. Ruppert, and G. Schütz. 1987. Genomic footprinting reveals cell type-specific DNA binding of ubiquitous factors. *Cell* 51:435-443.
5. Bender, T. P., C. B. Thompson, and W. M. Kuehl. 1987. Differential expression of *c-myc* mRNA in murine B lymphomas by a block to transcription elongation. *Science* 237:1473-1476.
6. Bentley, D. L., and M. Groudine. 1988. Sequence requirements for premature termination of transcription in the *c-myc* gene. *Cell* 53:245-256.
7. Bird, A. P. 1986. CpG-rich islands and the function of DNA methylation. *Nature (London)* 321:209-213.
8. Blobel, G. 1985. Gene gating: a hypothesis. *Proc. Natl. Acad. Sci. USA* 82:8527-8529.
9. Buschhausen, G., B. Wittig, M. Graessmann, and A. Graessmann. 1987. Chromatin structure is required to block transcription of the methylated herpes simplex virus thymidine kinase gene. *Proc. Natl. Acad. Sci. USA* 84:1177-1181.
10. Busslinger, M., J. Hurst, and R. A. Flavell. 1983. DNA methylation and the regulation of globin gene expression. *Cell* 34:197-206.
11. Cedar, H. 1988. DNA methylation and gene activity. *Cell* 53:3-4.
12. Chapman, B. S., and A. J. Tobin. 1979. Distribution of developmentally regulated hemoglobins in embryonic erythroid populations. *Dev. Biol.* 69:375-387.
13. Chelly, J., J.-P. Concordet, J.-C. Kaplan, and A. Kahn. 1989. Illegitimate transcription: transcription of any gene in any cell type. *Proc. Natl. Acad. Sci. USA* 86:2617-2621.
14. Chen, T. A., and V. G. Allfrey. 1987. Rapid and reversible changes in nucleosome structure accompany the activation, repression, and superinduction of murine fibroblast protooncogenes *c-fos* and *c-myc*. *Proc. Natl. Acad. Sci. USA* 84:5252-5256.
15. Chinsky, J. M., M.-C. Maa, V. Ramamurthy, and R. E. Kellems. 1989. Adenosine deaminase gene expression: tissue-dependent regulation of transcriptional elongation. *J. Biol. Chem.* 264: 14561-14565.
16. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294-5299.
17. Cleveland, D. W., M. A. Lopata, R. J. MacDonald, N. J. Cowan, W. J. Rutter, and M. W. Kirschner. 1980. Number and evolutionary conservation of α - and β -tubulin and cytoplasmic β - and γ -actin genes using specific cloned cDNA probes. *Cell* 20:95-105.
18. Cohen, R. B., and M. Sheffery. 1985. Nucleosome disruption precedes transcription and is largely limited to the transcribed domain of globin genes in murine erythroleukemia cells. *J. Mol. Biol.* 182:109-129.
19. de la Peña, P., and M. Zasloff. 1987. Enhancement of mRNA nuclear transport by promoter elements. *Cell* 50:613-619.
20. Dodgson, J. B., S. J. Stadt, O.-R. Choi, M. Dolan, H. D. Fischer, and J. D. Engel. 1983. The nucleotide sequence of the embryonic chicken β -type globin genes. *J. Biol. Chem.* 258:12685-12692.
21. Dolan, M., J. B. Dodgson, and J. D. Engel. 1983. Analysis of the adult chicken β -globin gene: nucleotide sequence of the locus, microheterogeneity at the 5'-end of β -globin mRNA, and aberrant nuclear RNA species. *J. Biol. Chem.* 258:3983-3990.
22. Dorbic, T., and B. Wittig. 1987. Chromatin from transcribed genes contains HMG17 only downstream from the starting point of transcription. *EMBO J.* 6:2393-2399.
23. Eick, D., and G. W. Bornkamm. 1986. Transcriptional arrest within the first exon is a fast control mechanism in *c-myc* gene

- expression. *Nucleic Acids Res.* **14**:8331–8346.
24. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**:6–13.
 25. Fodor, E. J. B., and P. Doty. 1977. Highly specific transcription of globin sequences in isolated reticulocyte nuclei. *Biochem. Biophys. Res. Commun.* **77**:1478–1485.
 26. Gariglio, P., M. Bellard, and P. Chambon. 1981. Clustering of RNA polymerase B molecules in the 5' moiety of the adult β -globin gene of hen erythrocytes. *Nucleic Acids Res.* **9**:2589–2598.
 27. Garner, M. M., G. Felsenfeld, M. H. O'Dea, and M. Gellert. 1987. Effects of DNA supercoiling on the topological properties of nucleosomes. *Proc. Natl. Acad. Sci. USA* **84**:2620–2623.
 28. Gerber-Huber, S., F. E. B. May, B. R. Westley, B. K. Felber, H. A. Hoshbach, A.-C. Andres, and G. U. Ryffel. 1983. In contrast to other *Xenopus* genes the estrogen-inducible vitellogenin genes are expressed when totally methylated. *Cell* **33**:43–51.
 29. Gilmour, D. S., and J. T. Lis. 1985. In vivo interactions of RNA polymerase II with genes of *Drosophila melanogaster*. *Mol. Cell. Biol.* **5**:2009–2018.
 30. Ginder, G. D., and J. D. McGhee. 1981. DNA methylation in the chicken adult β -globin gene: a relationship with gene expression, p. 191–201. In G. Stamatoyannopoulos and A. W. Nienhuis (ed.), *Organization and expression of globin genes*. Alan R. Liss, Inc., New York.
 31. Ginder, G. D., M. J. Whitters, and J. K. Pohlman. 1984. Activation of a chicken embryonic globin gene in adult erythroid cells by 5-azacytidine and sodium butyrate. *Proc. Natl. Acad. Sci. USA* **81**:3954–3958.
 32. Groudine, M., and H. Weintraub. 1980. Activation of cellular genes by avian RNA tumor viruses. *Proc. Natl. Acad. Sci. USA* **77**:5351–5354.
 33. Hay, N., and Y. Aloni. 1985. Attenuation of late simian virus 40 mRNA synthesis is enhanced by the agnoprotein and is temporally regulated in isolated nuclear systems. *Mol. Cell. Biol.* **5**:1327–1334.
 34. Humphries, S., J. Windass, and R. Williamson. 1976. Mouse globin gene expression in erythroid and non-erythroid tissues. *Cell* **7**:267–277.
 35. Hunter, E. 1979. Biological techniques for avian sarcoma viruses. *Methods Enzymol.* **58**:379–393.
 36. Hutchison, N., and H. Weintraub. 1985. Localization of DNase I-sensitive sequences to specific regions of interphase nuclei. *Cell* **43**:471–482.
 37. Iwamoto, S., F. Eggerding, E. Falck-Pederson, and J. E. Darnell. 1986. Transcription unit mapping in adenovirus: regions of termination. *J. Virol.* **59**:112–119.
 38. Jackson, V. 1987. Deposition of newly synthesized histones: new histones H2A and H2B do not deposit in the same nucleosome with new histones H3 and H4. *Biochemistry* **26**:2315–2325.
 39. Jantzen, K., H. P. Fritton, and T. Igo-Kemenes. 1986. The DNase I sensitive domain of the chicken lysozyme gene spans 24 kb. *Nucleic Acids Res.* **14**:6085–6099.
 40. Johnson, E. M., R. Sterner, and V. G. Allfrey. 1987. Altered nucleosomes of active nucleolar chromatin contain accessible histone H3 in its hyperacetylated forms. *J. Biol. Chem.* **262**:6943–6946.
 41. Kao, S.-Y., A. F. Calman, P. A. Luciw, and B. M. Peterlin. 1987. Anti-termination of transcription within the long terminal repeat of HIV-1 by *tat* gene product. *Nature (London)* **330**:489–493.
 42. Kelley, D. E., B. A. Pollok, M. L. Atchison, and R. P. Perry. 1988. The coupling between enhancer activity and hypomethylation of κ immunoglobulin genes is developmentally regulated. *Mol. Cell. Biol.* **8**:930–937.
 43. Keshet, I., J. Lieman-Hurwitz, and H. Cedar. 1986. DNA methylation affects the formation of active chromatin. *Cell* **44**:535–543.
 44. Kovsed, I., R. Reichel, and J. R. Nevins. 1987. Role of an adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control. *Proc. Natl. Acad. Sci. USA* **84**:2180–2184.
 45. Landes, G. M., and H. G. Martinson. 1982. Transcriptional properties of chick embryonic erythroid nuclei in vitro. *J. Biol. Chem.* **257**:11002–11007.
 46. Landes, G. M., B. Villeponteau, T. M. Pribyl, and H. G. Martinson. 1982. Hemoglobin switching in chickens: is the switch initiated post-transcriptionally? *J. Biol. Chem.* **257**:11008–11014.
 47. Law, R., M. D. Kuwabara, M. Briskin, N. Fasel, G. Hermanson, D. S. Sigman, and R. Wall. 1987. Protein-binding site at the immunoglobulin μ -membrane polyadenylation signal: possible role in transcription termination. *Proc. Natl. Acad. Sci. USA* **84**:9160–9164.
 48. Lawrence, J. B., R. H. Singer, and L. M. Marselle. 1989. Highly localized tracks of specific transcripts within interphase nuclei visualized by in situ hybridization. *Cell* **57**:493–502.
 49. Lawson, G. M., B. J. Knoll, C. J. March, S. L. C. Woo, M.-J. Tsai, and B. W. O'Malley. 1982. Definition of 5' and 3' structural boundaries of the chromatin domain containing the ovalbumin multigene family. *J. Biol. Chem.* **257**:1501–1507.
 50. Lois, R., and H. G. Martinson. 1989. Chicken globin gene transcription is cell lineage specific during the time of the switch. *Biochemistry* **28**:2281–2287.
 51. Maderious, A., and S. Chen-Kiang. 1984. Pausing and premature termination of human RNA polymerase II during transcription of adenovirus in vivo and in vitro. *Proc. Natl. Acad. Sci. USA* **81**:5931–5935.
 52. Mandel, J. L., and P. Chambon. 1979. DNA methylation: organ specific variations in the methylation pattern within and around ovalbumin and other chicken genes. *Nucleic Acids Res.* **7**:2081–2103.
 53. Mather, E. L., K. J. Nelson, J. Haimovich, and R. P. Perry. 1984. Mode of regulation of immunoglobulin μ - and δ -chain expression varies during B-lymphocyte maturation. *Cell* **36**:329–338.
 54. Mavilio, F., A. Giampaolo, A. Carè, G. Migliaccio, M. Calandrin, G. Russo, G. L. Pagliardi, G. Mastroberardino, M. Marinucci, and C. Peschle. 1983. Molecular mechanisms of human hemoglobin switching: selective undermethylation and expression of globin genes in embryonic, fetal, and adult erythroblasts. *Proc. Natl. Acad. Sci. USA* **80**:6907–6911.
 55. McReynolds, L. A., J. F. Catterall, and B. W. O'Malley. 1977. The ovalbumin gene: Cloning of a complete ds-cDNA in a bacterial plasmid. *Gene* **2**:217–231.
 56. Michalowsky, L. A., and P. A. Jones. 1989. Gene structure and transcription in mouse cells with extensively demethylated DNA. *Mol. Cell. Biol.* **9**:885–892.
 57. Mok, M., A. Maderious, and S. Chen-Kiang. 1984. Premature termination by human RNA polymerase II occurs temporally in the adenovirus major late transcriptional unit. *Mol. Cell. Biol.* **4**:2031–2040.
 58. Moreno, M. L., S. A. Chrysogelos, G. S. Stein, and J. L. Stein. 1986. Reversible changes in the nucleosomal organization of a human H4 histone gene during the cell cycle. *Biochemistry* **25**:5364–5370.
 59. Mukherjee, R., and G. R. Molloy. 1987. 5,6-Dichloro-1- β -D-ribofuranosylbenzimidazole inhibits transcription of the β -hemoglobin gene in vivo at initiation. *J. Biol. Chem.* **262**:13697–13705.
 60. Murray, E. J., and F. Grosfeld. 1987. Site specific demethylation in the promoter of human γ -globin gene does not alleviate methylation mediated suppression. *EMBO J.* **6**:2329–2335.
 61. Penefsky, H. S. 1977. Reversible binding of P_i by beef heart mitochondrial adenosine triphosphatase. *J. Biol. Chem.* **252**:2891–2899.
 62. Pribyl, T. A., and H. G. Martinson. 1988. Transcription termination at the chicken β^H -globin gene. *Mol. Cell. Biol.* **8**:5369–5377.
 63. Reeves, R. 1984. Transcriptionally active chromatin. *Biochim. Biophys. Acta* **782**:343–393.
 64. Roninson, I. B., and V. M. Ingram. 1983. Expression and partial sequence of the chicken β^H -globin gene. *J. Biol. Chem.* **258**:802–809.
 65. Sarkar, G., and S. S. Sommer. 1989. Access to a messenger

- RNA sequence or its protein product is not limited by tissue or species specificity. *Science* **244**:331-334.
66. **Shen, C.-K. J., and T. Maniatis.** 1980. Tissue-specific DNA methylation in a cluster of rabbit β -like globin genes. *Proc. Natl. Acad. Sci. USA* **77**:6634-6638.
67. **Smith, R. D., J. Yu, and R. L. Seale.** 1984. Chromatin structure of the β -globin gene family in murine erythroleukemia cells. *Biochemistry* **23**:785-790.
68. **Sneider, T. W.** 1980. The 5'-cytosine in CCGG is methylated in two eukaryotic DNAs and MspI is sensitive to methylation at this site. *Nucleic Acids Res.* **8**:3829-3840.
69. **Tanaka, K., E. Appella, and G. Jay.** 1983. Developmental activation of the H-2K gene is correlated with an increase in DNA methylation. *Cell* **35**:457-465.
70. **van der Ploeg, L. H. T., and R. A. Flavell.** 1980. DNA methylation in the human $\gamma\delta\beta$ -globin locus in erythroid and nonerythroid tissues. *Cell* **19**:947-958.
71. **Villeponteau, B., G. M. Landes, M. J. Pankratz, and H. G. Martinson.** 1982. The chicken β -globin gene region: delineation of transcription units and developmental regulation of interspersed DNA repeats. *J. Biol. Chem.* **257**:11015-11023.
72. **Villeponteau, B., M. Lundell, and H. Martinson.** 1984. Torsional stress promotes the DNase I sensitivity of active genes. *Cell* **39**:469-478.
73. **Villeponteau, B., and H. G. Martinson.** 1987. Gamma rays and bleomycin nick DNA and reverse the DNase I sensitivity of β -globin gene chromatin in vivo. *Mol. Cell. Biol.* **7**:1917-1924.
74. **Weber, J., W. Jelinek, and J. E. Darnell.** 1977. The definition of a large viral transcription unit late in AD2 infection of HeLa cells: mapping of nascent RNA molecules labeled in isolated nuclei. *Cell* **10**:611-616.
75. **Weintraub, H., H. Beug, M. Groudine, and T. Graf.** 1982. Temperature-sensitive changes in the structure of globin chromatin in lines of red cell precursors transformed by ts-AEV. *Cell* **28**:931-940.
76. **Weintraub, H., and M. Groudine.** 1976. Chromosomal subunits in active genes have an altered conformation. *Science* **193**:848-856.
77. **Weintraub, H., M. Groudine, S. Weisbrod, D. Riley, and I. Albanese.** 1979. Control of globin gene expression, p. 721-745. In G. Stamatoyannopoulos and A. W. Nienhuis (ed.), *Cellular and molecular regulation of hemoglobin switching*. Grune & Stratton, New York.
78. **Wood, W. I., and G. Felsenfeld.** 1982. Chromatin structure of the chicken β -globin gene region: sensitivity to DNase I, micrococcal nuclease, and DNase II. *J. Biol. Chem.* **257**:7730-7736.
79. **Wu, T.-C., and R. T. Simpson.** 1985. Transient alterations of the chromatin structure of sea urchin early histone genes during embryogenesis. *Nucleic Acids Res.* **13**:6185-6203.
80. **Yuan, D., A. C. Gilliam, and P. W. Tucker.** 1985. Regulation of expression of immunoglobulins M and D in murine B cells. *Fed. Proc.* **44**:2652-2659.