Fidelity of Globin Ribonucleic Acid Synthesis in Vitro by Isolated Nuclei: Asymmetric Gene Expression†

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ABSTRACT: Globin RNA synthesis in vitro by isolated erythroleukemic cell nuclei in the presence of a mercurinucleotide has been recently described (Orkin, S. H., and Swerdlow, P. S. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 2475–2479). The rationale for this approach to the study of eucaryotic gene expression is based on the premise that in vitro synthesis of RNA dependent on endogenous RNA polymerases within intact nuclei might prove more faithful than that reported in chromatin transcription systems where exogenous, nonhomologous RNA polymerases are added in excess. The validity of the assay system is dependent on the ability to purify specifically newly synthesized RNAs containing covalently bound mercury which are not contaminated with endogenous RNA sequences. In the study reported here the following criteria are satisfied in this nuclear system: (1) HgRNA transcripts synthesized by isolated nuclei have been purified greater than 95% free of contaminating, endogenous globin RNA sequences; (2) the appearance of globin RNA sequences in the purified transcripts is dependent on in vitro RNA synthesis and inhibited by actinomycin D, an inhibitor of DNA-directed RNA synthesis; and (3) globin RNA synthesis is completely asymmetric, i.e., no RNA complementary to globin RNA is detectable. Taken together, these data demonstrate the validity and fidelity of this assay of globin RNA synthesis in isolated nuclei. These results contrast those reported in chromatin transcription systems where the above criteria have not been satisfied. The nuclear system provides a reasonable alternative to other cell-free transcription systems for the study of eucaryotic gene expression in vitro.

Considerable attention has been devoted in recent years to the possibility of studying the expression of unique eucaryotic genes in cell-free transcription systems. The prototype experiment has employed complementary DNA probes (cDNAs)[1] to quantitate specific RNA sequences in putative transcripts prepared from the incubation of isolated chromatin of specialized tissues with exogenous *E. coli* RNA polymerase (Axel et al., 1973; Gilmour and Paul, 1973). Results have been presented from a number of laboratories which seemingly demonstrate the synthesis of globin (Axel et al., 1973; Gilmour and Paul, 1973; Steggles et al., 1974; Barrett et al., 1974; Crouse et al., 1976), immunoglobulin light chain (Smith and Huang, 1976), histone (Stein et al., 1975), and ovalbumin (Harris et al., 1976; Towle et al., 1977) RNA sequences in chromatin transcription systems. Difficulties in these experiments, however, often included the failure to separate endogenous RNA from newly synthesized material and the synthesis of RNA sequences complementary to RNA species normally present (Zasloff and Felsenfeld, 1977; Wilson et al., 1975).

The availability of mercurinucleotides (Dale et al., 1974, 1975; Dale and Ward, 1975) permits newly synthesized RNA to be purified specifically and has allowed closer examination of chromatin transcription experiments. Under the conditions of chromatin transcription generally employed, *E. coli* RNA polymerase can utilize endogenous RNAs as templates for the synthesis of complementary RNAs (antisense RNAs) (Zasloff and Felsenfeld, 1977). When transcripts of duck reticulocyte chromatin were prepared in the presence of a mercurinucleotide and purified by affinity chromatography without prior heat denaturation, globin sequences were readily detected in the mercurated RNA. The apparent synthesis of globin sequences, however, was artefactual as the newly synthesized, mercurated RNA was in fact present in duplex structure with endogenous globin RNA (Zasloff and Felsenfeld, 1977). Mercurated transcripts prepared by affinity chromatography after heat denaturation were essentially devoid of globin sequences. The extent to which the apparent synthesis of specific RNA sequences in chromatin transcription experiments of others is subject to this serious artefact is unknown, but quite likely considerable. It is important to emphasize that the artefact of these experiments is due to transcription of RNA by *E. coli* RNA polymerase and not due to the use of a mercurinucleotide. In view of these recent developments, conclusions drawn from in vitro chromatin transcription experiments must be reevaluated critically.

As an alternative approach to the study of eucaryotic gene expression, we have recently focused on RNA synthesis in isolated cell nuclei (Orkin and Swerdlow, 1977). Our work is founded on the premise that in vitro synthesis of RNA dependent on endogenous RNA polymerases within intact nuclei might prove more faithful than that in chromatin systems where nuclear integrity is disrupted and excess polymerase is added exogenously. When nuclei of erythroleukemic cells induced to differentiate along an erythroid pathway in tissue culture were utilized, globin RNA sequences were detected in RNA transcripts containing a mercurinucleotide at a much higher concentration than in similar transcripts from nuclei of uninduced cells (Orkin and Swerdlow, 1977). These observations indicated that differentiation in these cells was as-

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[1] Abbreviations used: HgRNA, mercurated RNA; cDNA, DNA probe complementary to globin RNA; ccDNA, DNA probe complementary to globin cDNA; Tris, tris(hydroxymethyl)aminoethane; EDTA, ethylenediaminetetraacetic acid; Hepes, hydroxyethylpiperazineethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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sociated with increased expression, i.e., transcriptional activation, of the globin genes. Since initiation of new RNA chains by RNA polymerase II appears inefficient in current in vitro systems (Gilboa et al., 1977), the presence of globin RNA sequences in the mercurated RNA fraction most likely reflected predominantly elongation of nascent globin transcripts in the isolated nuclei.

In our initial studies mercurated RNA transcripts were purified by chromatography on sulfhydryl-Sepharose under conditions meant to minimize aggregates but not disrupt duplex structures. If antisense globin RNA sequences were present, our results might have been attributable to the artefact noted above in chromatin systems. Here I examine several aspects of the nuclear transcription system, particularly the purification of the mercurated RNA transcripts, their template dependence, and their strandedness. The results demonstrate the validity and fidelity of the nuclear system and contrast the findings of chromatin transcription experiments.

Experimental Procedure

Cell Culture. Erythroleukemic cells of the long-term passage stock of the clone T3C12 were grown as before (Orkin and Swerdlow, 1977). Induction of erythroid differentiation and globin expression was achieved by passage into 1.5% dimethyl sulfoxide for 72 h.

Nuclear RNA Synthesis. Nuclei were purified from either control or induced erythroleukemic cells by treatment with Triton X-100 and centrifugation through a sucrose cushion (Orkin and Swerdlow, 1977). For RNA synthesis isolated nuclei (approximately 5×10^7 cells per mL) were incubated in 50 mM Hepes, pH 8.0, 5 mM $Mg(OAc)_2$, 1 mM $MnCl_2$, 150 mM KCl, 12.5% glycerol, 4 mM phosphoenolpyruvate, pyruvate kinase (3 U per mL), 0.185 mM ATP and GTP, 0.037 mM [^{3}H]UTP (New England Nuclear, final specific activity 3 Ci/mmol), 0.185 mM 5′-mercuricytidine triphosphate (Hg-CTP) (P-L Biochemicals) or CTP, and 10 mM thioglycerol at 23 °C with intermittent shaking for either 5 or 15 min. RNA was extracted as previously described (Orkin and Swerdlow, 1977).

Affinity Purification of HgRNA. Newly synthesized, mercurated RNA (HgRNA) was purified by chromatography on sulfhydryl-Sepharose 6B (Dale and Ward, 1975) prepared by the method of Cuatrecasas (1970). Each batch of column material was tested for its ability to bind HgRNA and its level of nonspecific adsorption of nonmercurated RNA. Characterization of the columns is essential as some batches of sulfhydryl-Sepharose have for unknown reasons unacceptably high nonspecific adsorption (0.5–1%). With such unacceptable batches of column material, maneuvers such as the addition of dimethyl sulfoxide washes (Crouse et al., 1976) did not appreciably reduce this contamination (Orkin, S. H., unpublished). Two milliliters of sulfhydryl-Sepharose was packed and washed with 20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA, and 0.1 M β-mercaptoethanol prior to extensive washing with buffer lacking mercaptan. Before application of a sample, the column was washed with buffer containing 0.5% sodium dodecyl sulfate ($NaDodSO_4$). Ethanol-precipitated RNA samples were resuspended in 10 mM Tris-HCl, pH 7.5. $NaDodSO_4$ was added to 0.5% final concentration and the samples were either heated for 5 min at 65 °C or heat-denatured for 2 min in a preheated tube at 100 °C. Samples were quickly cooled on ice. After addition of NaCl to 0.1 M, samples were applied slowly to the column (15–20 min), which was then washed successively with 100 mL of buffer containing $NaDodSO_4$ and then 250 mL of buffer minus $NaDodSO_4$. HgRNA was eluted with buffer containing 0.1 M β-mercaptoethanol and ethanol precipitated with 100–200 μg of carrier *E. coli* or yeast tRNA. In some experiments the affinity column was washed at 50 °C with low salt buffer (10 mM Tris-HCl, pH 7.5) containing 0.5% $NaDodSO_4$ prior to the usual column wash minus $NaDodSO_4$ at ambient temperature. Under these conditions the globin sequence content of HgRNA isolated from induced cell nuclei was not different from that in HgRNA purified in the manner described above (Orkin, S. H., unpublished).

Preparation of Globin cDNA and ccDNA. ^{32}P-labeled globin cDNA (specific activity approximately 1.5×10^8 cpm/μg) was prepared as previously described (Orkin and Swerdlow, 1977). Antisense globin sequences can be assayed by hybridization with a DNA probe, designated ccDNA (Orkin, 1977), synthesized from globin cDNA as a template. The preparation of this probe has been described completely elsewhere (Orkin, 1977). In brief, mouse globin cDNA served as a template for avian myeloblastosis virus DNA polymerase. The resulting double-stranded DNA was treated with S_1 nuclease and then heat-denatured in the presence of a vast excess of globin RNA. cDNA and ccDNA strands were separated by chromatography on hydroxylapatite. The ccDNA probe hybridized to globin cDNA with a rate and extent of reaction similar to that observed in the hybridization of cDNA with mRNA (Orkin, 1977). No hybridization with globin RNA was observed.

Molecular Hybridization. Nuclear RNA–cDNA hybridization for the quantitation of steady-state, endogenous globin RNA was performed as described for quantitation of globin RNA in cytoplasmic RNA (Orkin et al., 1975). Hybridization of ^{3}H-labeled HgRNA transcripts with ^{32}P-labeled globin cDNA or ^{32}P-labeled ccDNA was performed at 65 °C for 24–36 h in 10 μL of total volume containing 0.6 M NaCl, 20 mM Tris-HCl, pH 7.5, 5 μg of additional *E. coli* tRNA, 1 μg of calf thymus DNA, 0.01% $NaDodSO_4$, varying amounts of the transcripts, and 3–5 pg of ^{32}P-labeled cDNA or ^{32}P-labeled ccDNA. Hybridization of the ^{32}P-labeled probes was assessed by digestion with S_1 nuclease (Orkin and Swerdlow, 1977). Control experiments demonstrated that there were no inhibitors of S_1 nuclease in the HgRNA preparations. Demercuration of the HgRNA samples with β-mercaptoethanol prior to or during molecular hybridization did not appreciably affect the hybridization observed.

Quantitation of Globin Sequences in Total Nuclear RNA. Nuclei prepared for standard in vitro incubations were subjected to phenol extraction precisely as described for the isolation of RNA after in vitro synthesis. The recovered nucleic acids were treated with RNase-free DNase (Worthington Biochemicals), phenol extracted again, and ethanol precipitated. The precipitated RNA was chromatographed on Sephadex G-50 in 20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA to remove oligodeoxynucleotides. The excluded material was pooled and ethanol precipitated. Its globin sequence content was assessed by molecular hybridization with globin cDNA as described above. Control experiments demonstrated that no globin sequences were detectable by hybridization in the oligodeoxynucleotide fraction. Thus, all endogenous globin RNA sequences were present in the excluded fraction.

Results

Purification of Mercurated RNA Transcripts. RNA synthesis in vitro within isolated nuclei may include elongation and completion of nascent chains as well as reinitiation of RNA chains. Previous evidence suggested that chain elongation predominates in incubated nuclei (Smith and Huang, 1976; Ernest et al., 1976). Very recently Gilboa et al. (1977) dem-

onstrated that initiation by RNA polymerase II in isolated erythroleukemia cell nuclei is inefficient or does not seem to occur. However, it should be noted that their method of measuring initiation tended to bias against detection of initiation of larger RNA species (Gilboa et al., 1977). RNA synthesized in vitro in the presence of a mercurinucleotide results in the formation of RNA molecules containing covalently bound mercury. These RNAs are referred to here as mercurated RNA transcripts, independent of whether they are elongated, nascent RNAs or reinitiated molecules. Preexisting RNAs which are not nascent are designated endogenous RNAs.

Contamination of newly synthesized, mercurated RNA transcripts with endogenous globin RNA sequences might be of two sorts: first, low level nonspecific adsorption on sulfhydryl-Sepharose of nonmercurated, endogenous RNA present in vast excess might result in significant contamination of the specifically bound, mercurated RNA with endogenous globin sequences; and, second, endogenous globin RNA sequences might be purified in duplex structures with antisense RNA synthesized in vitro either from RNA templates (Zasloff and Felsenfeld, 1977) or from aberrant strand selection for transcription. Reconstruction and RNA synthesis inhibition experiments were performed to eliminate the first possibility and establish that the hybridizable globin sequences in the purified mercurated transcripts were indeed due to newly synthesized RNA. To investigate the latter possibility, transcripts were heat denatured prior to affinity chromatography and also assayed directly for the presence of antisense globin RNA using a DNA probe complementary to antisense RNA.

When nonmercurated RNA synthesized by nuclei of induced erythroleukemic cells was purified in "mock" fashion on sulfhydryl-Sepharose, the mercaptan eluate contained no globin sequences by molecular hybridization (Orkin and Swerdlow, 1977). This control experiment indicated that contamination with endogenous globin sequences was not responsible for the hybridization to globin cDNA in mercurated RNA transcripts purified in similar fashion. This kind of experiment, however, does not address the theoretical and observed (Konkel and Ingram, 1977) possibility of aggregation of nonmercurated RNA with mercurated RNA bound to the affinity column and subsequent contamination of the transcripts with endogenous sequences. To determine quantitatively the level of contamination due either to aggregation of nonmercurated RNA with mercurated RNA or nonspecific adsorption to the column itself, a series of reconstruction experiments was performed. Mercurated RNA was synthesized in vitro using nuclei of uninduced erythroleukemic cells. Such transcripts contain only a low concentration of globin sequences (Orkin and Swerdlow, 1977). Prior to affinity chromatography the sample was divided in thirds, two parts of which received exogenous globin RNA (24 ng or 650 ng). The samples were applied to sulfhydryl-Sepharose columns after disaggregation in low salt at 65 °C in 0.5% $NaDodSO_4$ and the mercurated RNA was eluted as usual. The recovered mercurated RNAs were compared in their hybridization to globin cDNA (Figure 1). The hybridization of the sample to which 24 ng of globin RNA had been added prior to the chromatography was nearly identical with that of the transcript to which no exogenous globin RNA had been added. However, addition of a much larger content of globin RNA (650 ng) to the sample prior to chromatography resulted in appreciable globin sequences in the purified RNA, equivalent to that seen in mercurated RNA transcripts of induced cell nuclei (Figure 1). By reference to a standard curve for hybridization of pure globin RNA with globin cDNA probe, it was evident that 0.02% of the exogenously added globin RNA sequences had

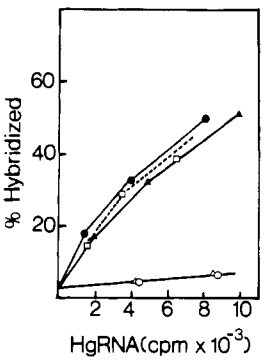

FIGURE 1: Hybridization of HgRNA transcripts with globin cDNA. Nuclei (9×10^7) of uninduced cells were incubated 15 min in vitro to prepare HgRNA transcript (approximately 300 synthesized RNA), which was divided into three parts. The following amount of globin RNA was added to each third before disaggregation of the samples in 0.5% $NaDodSO_4$, 10 mM Tris-HCl, pH 7.5, at 65 °C for 5 min and purification by affinity chromatography: none (○—○), 24 ng (△—△), or 650 ng (□- - -□). The percent ^{32}P-labeled globin cDNA hybridized was assessed vs. the input of ^3H-labeled HgRNA for each sample. In the transcript to which 650 ng of globin had been added, 125 pg of globin RNA was present in the total material recovered from the affinity column. Thus, contamination with endogenous globin RNA was 0.02%. Nuclei (7.4×10^7) of cells induced for 72 h with dimethyl sulfoxide were used in parallel to synthesize HgRNA transcript (approximately 120 ng of synthesized RNA) of induced nuclei. Prior to affinity chromatography, the sample was divided in half. One portion was heated to 65 °C as above (●—●); the remaining portion was heat-denatured and quickly cooled before the chromatography (▲—▲). The 20% difference in hybridization of these latter HgRNAs with the cDNA is insignificant in view of the data on direct measurement of antisense RNA shown in Figure 4. The amount of globin RNA present endogenously in each half of the induced nuclei can be calculated as follows: number of nuclei × RNA/nucleus × % globin RNA in total nuclear RNA = 3.7×10^7 nuclei × 3 pg/nucleus × 0.012% = 13.3 ng of globin RNA.

contaminated the purified, mercurated transcript. This experiment was repeated as well with the addition of exogenous globin RNA prior to phenol extraction of the isolated nuclei to rule out aggregation of RNAs even more definitively (Konkel and Ingram, 1977). Less than 0.01% of the exogenously added globin RNA appeared in the mercurated transcript.

The low level of nonspecific contamination (0.02% or less) was insignificant as the addition of the smaller amount of globin RNA (24 ng) slightly exceeded the globin RNA content present endogenously in induced cell nuclei in typical nuclear transcription experiments. To establish this, total nuclear RNA was isolated from an aliquot of the induced cell nuclei used in the transcriptions shown in Figure 1. The concentration of globin RNA sequences in the nuclear RNA was determined by hybridization to globin cDNA (Figure 2). The induced cell nuclei contained on average 3 pg of RNA per nucleus, 0.012% of which was globin (see legend). Uninduced nuclei had markedly less globin RNA present endogenously. For the number of nuclei employed in the transcriptions shown in Figure 1, only 13 ng of globin RNA was present endogenously in the induced nuclei. Thus, the addition of 24 ng of globin RNA to mercurated transcripts of uninduced nuclei was somewhat greater than the amount of endogenous globin RNA present in the number of induced nuclei studied. On average, induced cell nuclei at 72 h of induction with dimethyl sulfoxide contained about 1000 molecules of globin RNA, an estimate quite similar to that obtained by others (Gilmour et al., 1974). Since the addition of a nearly 50-fold excess of globin RNA over that present as endogenous RNA mimicked the hybridization to cDNA seen in transcripts from nuclei of induced cells, the mercurated transcripts were about 98% pure. Only

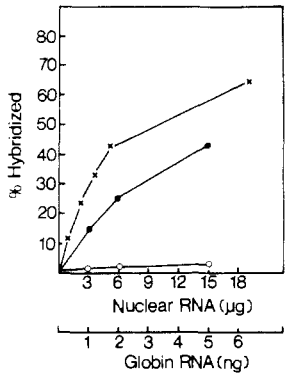

FIGURE 2: Endogenous globin RNA content of erythroleukemic cell nuclei. Total nuclear RNA was prepared from both uninduced and induced cell nuclei as described in Experimental Procedures. Hybridization of these RNAs (uninduced nuclear RNA, O—O; induced nuclear RNA, ●—●) with ^{32}P-labeled globin cDNA was compared with a standard curve for the hybridization of pure globin RNA (×—×). For the RNA from induced nuclei, 6 μg of nuclear RNA hybridized to the same extent as 750 pg of pure globin RNA. Thus, approximately 0.012% of the endogenous RNA contained globin sequences. Uninduced cell nuclei had at least 30-fold less globin sequences.

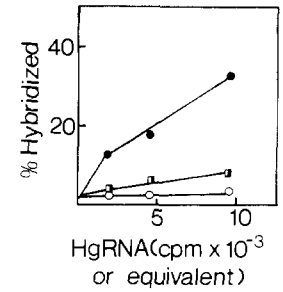

FIGURE 3: Template dependence. Four reactions each containing 3×10^7 nuclei of induced cells were incubated 15 min in vitro with the following specific conditions: complete incubation mixture with HgCTP present; complete mix with both HgCTP and actinomycin D (10 μg/mL) present; mix with HgCTP present but ATP, GTP omitted; and complete mix with CTP present in place of HgCTP. Transcripts were purified by affinity chromatography without heat denaturation and the eluted material was precipitated with carrier tRNA. The percent hybridization of ^{32}P-labeled globin cDNA was assessed vs. the input of each purified transcript. For the transcript from the complete mix with HgCTP present (●—●) the input RNA was quantitated by trichloroacetic acid precipitable radioactivity in the ^{3}H-labeled HgRNA as usual. To allow direct comparison of the relative globin content of all transcripts, equivalent percentages of the total transcripts of the other incubations were used in the hybridization reactions. Transcripts are as follows: + HgCTP, + actinomycin D and + HgCTP, − ATP, − GTP (□—□); CTP in place of HgCTP (O—O). This representation of the data avoids complexities introduced by alteration in the specific activities of the RNAs from the inhibited reactions due to diminished elongation of nascent RNA chains. The percent yields during purification of these transcripts were equal. Similar reduction of the appearance of globin sequences in the HgRNA fraction by actinomycin D was observed in three similar transcription experiments.

about 2% of the hybridizable globin sequences could be due to contaminating endogenous globin RNA.

Direct confirmation of these results was obtained in a complementary sort of control experiment in which nonmercurated in vitro synthesized RNA from nuclei of induced cells was applied to the affinity column after the addition of mercurated *E. coli* transfer RNA (see below). The recovered mercurated RNA was again not significantly contaminated by endogenous globin sequences. Furthermore, control nuclei were incubated in the presence of HgCTP and phenol extracted after the addition of an equivalent number of induced, nonincubated nuclei. The recovered HgRNA did not contain hybridizable globin sequences in excess of that observed in HgRNA prepared from incubated control nuclei. Taken together these data provide evidence that the purified mercurated RNA transcripts were essentially free of significant, contaminating endogenous sequences. On average, 20–60 pg of hybridizable globin sequences were present per 10^3 cpm (~10 ng of synthesized RNA) of HgRNA purified from a 15-min incubation of nuclei in vitro.

To investigate whether mercurated transcripts were contaminated by endogenous RNA present in duplex structure, transcripts were first heat denatured prior to application to the sulfhydryl-Sepharose column. Heat denaturation did not appreciably affect the percentage of HgRNA adsorbed by the column (data not shown). When samples of denatured transcripts were compared with native transcripts with respect to hybridization to globin cDNA, no significant difference was observed (Figure 1). This indirect experiment suggested that antisense globin RNA, if present, represented a minor component. The direct search for antisense globin RNA (see below) completely substantiated this conclusion, and further demonstrated that antisense globin RNA was undetectable. Hybridizable globin sequences in the mercurated transcripts then do not represent endogenous sequences isolated in duplex structure with in vitro synthesized antisense globin RNA.

Template Dependence. A critical point to establish is that the appearance of globin RNA sequences in the mercurated transcript fraction actually requires DNA-template directed RNA synthesis. In chromatin experiments bulk RNA synthesis may be inhibited to a marked degree with actinomycin D without appreciable effect on the artefactual appearance of globin sequences in undenatured transcripts, because the aberrant transcription on the RNA template is resistant to the antibiotic (Zasloff and Felsenfeld, 1977). Also, adventitious mercuration of endogenous RNA by HgCTP during the incubation of nuclei or subsequent phenol extraction of the RNA would not have been detected in the experiments presented above. A priori this latter possibility seemed remote in view of the specific conditions required for in vitro mercuration of polynucleotides (Dale et al., 1975). To address these issues directly, in vitro RNA synthesis in nuclei of induced erythroleukemic cells was inhibited by either omission of ribonucleoside triphosphates or addition of actinomycin D (10 μg per mL). RNA synthesis was inhibited 80% by addition of actinomycin D and 93% by omission of ATP and GTP during a 15-min incubation of the nuclei. As expected for faithful RNA synthesis on a DNA template, globin sequences were markedly reduced (approximately 90%) by either means of inhibition of total RNA synthesis (Figure 3). Included in the data are hybridization results for nonmercurated RNA transcripts purified in parallel. Contamination with endogenous sequences was 3% or less. The residual globin sequences found in the samples from the inhibited incubations may reflect the addition of one or a few mercurated nucleotides to a nascent globin chain prior to full inhibition by actinomycin D or prior to the necessity for the addition of one of the lacking ribonucleoside triphosphates to a growing RNA chain. Nevertheless, the results demonstrate that the appearance of globin RNA sequences in the HgRNA transcripts depends on RNA synthesis in the presence of all four ribonucleoside triphosphates on a DNA template.

Asymmetric Transcription of Globin Genes. The above experiments established the validity of the assay system for newly synthesized globin RNA and suggested that antisense globin RNA sequences were not present at high levels. To search quantitatively for possible antisense globin sequences,

mercurated RNA transcripts were incubated with a DNA probe specific for sequences complementary to globin RNA (Orkin, 1977). This probe, designated ccDNA, hybridized to globin cDNA, but not globin RNA, with a rate and extent very similar to that of globin cDNA with globin RNA. Mercurated transcripts were assayed in parallel with both cDNA and ccDNA probes to assess the relative amount of sense and antisense sequences. To ensure that rapidly synthesized and degraded antisense sequences might not be missed, transcripts from both 5- and 15-min incubations of nuclei were examined. RNA complementary to globin RNA was not detected in the mercurated transcripts of nuclei incubated for either time (Figure 4). This was observed whether or not the transcript was heat denatured prior to affinity chromatography (not shown). A control experiment which demonstrated that the sense RNA detected was not merely endogenous contamination is presented (Figure 4B). Since globin RNA would compete with globin ccDNA for hybridization with hypothetical antisense globin RNA, reconstruction experiments were performed to be certain that small amounts of antisense RNA would have been detected in such experiments. Antisense sequences added exogenously as globin cDNA were readily detected in reconstruction experiments at an input nearly equivalent to the concentration of globin RNA in the sample of mercurated RNA (data not shown). Thus, the absence of hybridization of the HgRNA transcripts to the ccDNA reflected the absence of antisense RNA sequences. Globin RNA synthesis by isolated nuclei is completely asymmetric.

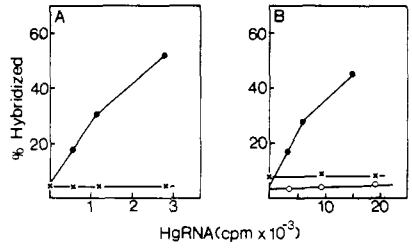

FIGURE 4: Absence of antisense globin RNA sequences in HgRNA transcripts. Nuclei of induced cells were incubated either 5 (A) or 15 (B) min in vitro to prepare RNA transcripts. The percent hybridization of ^{32}P-labeled globin cDNA (●—●) or ^{32}P-labeled globin ccDNA (X—X), each with a specific activity of 1-1.5 × 10^8 cpm/μg, is plotted vs. the amount of purified ^{3}H-labeled HgRNA transcript added. A sample of nonmercurated transcript from the 15-min incubation was also prepared. Three hundred nanograms of in vitro mercurated *E. coli* tRNA (Dale et al., 1975), an amount equal to the HgRNA synthesized in the parallel reaction, was added to the nonmercurated transcript prior to its purification. Aliquots of the material recovered from the affinity column equivalent to those used for the HgRNA sample were assayed for hybridization with ^{32}P-labeled globin cDNA (○—○). As noted previously (Orkin and Swerdlow, 1977), for a given number of counts per minute of ^{3}H-labeled HgRNA transcript, more hybridization with the ^{32}P-labeled globin cDNA is observed with transcript from the shorter in vitro incubation of isolated nuclei. This apparent increasing overall specific activity of globin sequences is due to continued RNA synthesis during the incubation. The total globin sequence contents of HgRNAs isolated from 5- and 15-min incubations of nuclei in vitro are not significantly different (Orkin, S. H., unpublished). The lack of net accumulation of globin sequences with time is consistent with chain elongation of nascent globin transcripts or lability of newly synthesized globin sequences. In the experiments shown here samples were not heat denatured prior to affinity chromatography, but were denatured at time zero of the molecular hybridization reaction.

Discussion

The in vitro nuclear RNA synthesizing system we have described (Orkin and Swerdlow, 1977), as shown in this paper, satisfies the following criteria: (1) HgRNA transcripts can be purified essentially free of contaminating endogenous globin sequences and therefore reflect in vitro synthesized material; (2) the appearance of globin sequences in the transcripts is dependent on in vitro RNA synthesis, and is inhibited by actinomycin D, an inhibitor of DNA-directed RNA synthesis; and (3) globin RNA synthesis is completely asymmetric—no antisense RNA is present. Taken together these observations eliminate artefacts which would result in the detection of globin sequences in purified transcripts. The assay of newly synthesized globin RNA in isolated nuclei is both valid and seemingly faithful.

Clearly these are minimum criteria which should be met by a cell-free, in vitro transcription system. Ultimately one would aim to demonstrate correct initiation and termination of RNA sequences and normal responses to authentic gene regulators. Whether these latter criteria can be satisfied in nuclear synthesizing systems is uncertain as the majority of RNA synthesis appears to consist of RNA chain elongation rather than initiation of new RNA chains. Indirect experiments in this system suggest that the globin RNA synthesis observed is almost exclusively due to elongation and completion of nascent globin RNA chains (Orkin, S. H., unpublished). Nevertheless, the criteria already met by this system establish its usefulness in studying relative rates of globin gene expression in nuclei obtained from cells in varying states of erythroid differentiation.

The nuclear system contrasts sharply with chromatin transcription systems reported in the literature. When chromatin transcripts have been assayed for antisense RNA sequences, they have been observed (Wilson et al., 1975; Honjo and Reeder, 1974; Towle et al., 1977). Apparently a large part of such sequences, if not all, may arise from utilization of endogenous RNAs as templates (Zasloff and Felsenfeld, 1977). Whatever the origin of such sequences, their presence is a reflection of in vitro artefacts probably related to the use of excess, exogenous *E. coli* RNA polymerase or possibly to unknown perturbations in chromatin structure. Whether, in fact, there is selective expression of unique genes in chromatin in these experiments must be carefully reexamined. Chromatin transcription experiments reported to date do not satisfy the minimum criteria cited above. In other experiments (Reff, M., Orkin, S. H., and Davidson, R., unpublished), we have confirmed the work of Zasloff and Felsenfeld (1977) using erythroleukemic (Friend) cell chromatin and exogenous *E. coli* RNA polymerase. In view of these observations it would be prudent to reevaluate previous Friend cell chromatin experiments (Gilmour et al., 1974).

The nuclear RNA synthesizing system provides an alternative to chromatin systems for the study of unique gene expression in vitro at this time. Isolated nuclei are most suitable for measuring the relative rates of gene expression in differentiating cells and following the fate of newly synthesized material under cell-free conditions. They could serve as a test system to determine those factors which might be lacking in the chromatin systems generally employed. If initiation of gene expression and its control are to be fruitfully investigated with isolated nuclei, conditions must be established that permit extensive reinitiation of RNA chains transcribed from unique gene loci. Whether the addition of homologous RNA polymerases (Sklar and Roeder, 1977) or cytoplasmic factors to isolated nuclei will achieve these goals is uncertain, but worthwhile exploring.

Ultimately, however, a more complete understanding of gene expression in eucaryotic cells may necessitate development of a chromatin transcription system in which component parts can be manipulated individually. If this could be ac-

complished without compromising the fidelity of gene expression in vitro, it would be the system of choice for further investigations.

Acknowledgments

I thank Ms. Domenica Paci for excellent technical assistance, Dr. David G. Nathan for continued support and criticism, and Ms. Catherine Lewis for preparation of this manuscript.

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