Synthesis of Globin RNA in Enucleated Differentiating Murine Erythroleukemia Cells

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Abstract. In an earlier report (Volloch, V., 1986, *Proc. Natl. Acad. Sci. USA.*, 83:1208–1212) we had presented evidence for the occurrence of the cytoplasmic synthesis of globin mRNA and of RNA complementary to globin mRNA which differed from DNA-dependent transcription by its insensitivity to actinomycin D. In this paper, we describe the use of enucleated differentiating mouse erythroleukemia cells to demonstrate directly the occurrence of cytoplasmic synthesis of both positive- and negative-strand globin RNA. For this purpose, we developed an enucleation procedure which yielded pure cytoplasts from differentiated mouse erythroleukemia cells in the absence of cytochalasin B and selectively permeabilized the cytoplasts to small molecules by treatment with dextran sulfate and saponin. The permeabilized cytoplasts incorporated [^{3}H]dUTP into positive- and negative-strand globin RNA and experiments with mercurated nucleotide substrate suggested that this process involved de novo RNA synthesis rather than limited terminal nucleotide addition. Globin RNA synthesis required Mg^{++}, was inhibited by Mn^{++}, and was unaffected by the addition of Zn^{++}. Studies of its response to inhibitors of DNA-dependent RNA synthesis showed that it differed from that process in its insensitivity to actinomycin D and α-amanitin, but that like many other macromolecular biosynthetic reactions it was inhibited by rifamycin AF/ABDP and aurintricarboxylic acid. These observations provide additional evidence for the occurrence of cytoplasmic RNA-dependent RNA synthesis in differentiated cells and show permeabilized enucleated cells to be a useful experimental system for further studies of the characteristics of that process.

MURINE hematopoietic precursor cells can be transformed by Friend virus to yield erythroid cell lines, murine erythroleukemia (MEL)[1] cells, which are blocked at a relatively advanced stage in their differentiation pathway (18). This block in erythroid differentiation is phenotypically reversible in vitro. When cultured in the presence of an inducer such as hexamethylenebisacetamide or DMSO, these cells become committed to a terminal differentiation program (13) and undergo a number of changes which culminate in the loss of the nucleus (31, 32) and include loss of the capacity for cell division (10), accumulation of the heme synthetic enzymes (33), alterations in membrane proteins composition (22), and most characteristically, massive synthesis and accumulation of globin mRNA and hemoglobin. A major factor responsible for the increase in hemoglobin synthesis may be the increase in the rate of transcription of the corresponding globin gene (25). However, the involvement of a second mechanism is suggested by the recent report (30) that RNA-dependent RNA synthesis occurs in the cytoplasm of MEL cells using globin mRNA as a template and markedly increases during induction. This mechanism is differentiation specific and may be an important determinant of globin mRNA levels, as indicated by observations regarding the kinetics of globin RNA synthesis, localization of newly synthesized globin RNA, its size and distribution among different RNA fractions, distribution of radioactive nucleotides along the globin RNA molecule, drug sensitivity of synthesis, and presence of negative-strand globin RNA. Nevertheless, many questions remain unanswered and additional evidence must be sought. Accordingly, we resorted to the use of enucleated cells or cytoplasts, which allows the separation of nuclear and cytoplasmic processes and avoids the need to use drugs such as actinomycin D, which may give rise to artifacts. A further refinement of this approach involved selective permeabilization of enucleated cells to allow manipulation by the introduction of molecules of interest such as nucleotide substrates and inhibitors. As described in this paper, the use of enucleated cells and their permeabilization allowed more detailed characterization of cytoplasmic RNA-dependent synthesis of globin mRNA.

Materials and Methods

Cell Culture

MEL cells (line 745, clone PC4-12-9) were cultured in DME with 15% FCS

1. *Abbreviation used in this paper:* MEL, murine erythroleukemia.

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(Gibco, Grand Island, NY). Cells were induced to differentiate by addition of hexamethylenebisacetamide (4 mM; Aldrich Chemical Co., Milwaukee, WI) in 3% BSA or 2% Ficoll 400.

Preparation of Enucleated Cells (Cytoplasts)

Cytoplasts of differentiating MEL cells were prepared as described elsewhere (34), except that no cytochalasin B was used in the enucleation procedures. A discontinuous density gradient was prepared in ultracentrifuge tubes from solutions of Ficoll 400 (Pharmacia Fine Chemicals, Piscataway, NJ) in MEM (Gibco, Grand Island, NY). The Ficoll stock solution (50% wt/wt in water, autoclaved and stored at −20°C) was first diluted with an equal volume of double-strength MEM and then with MEM to the desired concentrations. The appropriate solutions were incubated in a CO_2 incubator, to a pH of 7.2–7.6, and then dispensed to yield a discontinuous gradient consisting of 25, 19, 18, 17, 16, 15, 12.5, and 10% Ficoll in MEM. The gradients were incubated for 12–18 h at 37°C before use, and then overlaid with cells in 10% Ficoll, followed by 2% Ficoll. After centrifugation at 25,000 rpm for 60 min at 30°C, cytoplasts were collected from the appropriate banding interface (16–17% Ficoll) with a syringe, diluted into fresh medium, pelleted by low speed centrifugation, resuspended in fresh medium, and diluted to the desired concentration for further studies. For preparation of the cytoplasts from uninduced MEL cells or from L cells, cytochalasin B was added to all fractions of the gradient to a final concentration of 10 μg/ml (from stock solution of 2 mg/ml in DMSO). The efficiency of enucleation was determined by staining with Feulgen (DNA-specific) stain and found to be greater than 99.9%.

Permeabilization of Cytoplasts of Differentiating MEL Cells

All cell manipulations were carried out at 4°C. Cytoplasts were collected by centrifugation and resuspended in a buffer containing 50 mM Hepes, pH 7.6, 80 mM NaCl, 20 mM KCl, 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and 2% Ficoll (Buffer A) and washed twice by centrifugation and resuspension in that buffer. The suspension was adjusted to 10^8 cytoplasts per ml and supplemented with an equal volume of dextran sulfate (200 μg/ml) plus saponin (200 μg/ml) in Buffer A. Permeabilization was monitored by the uptake of the vital dye erythrosin B. A small sample of suspension was incubated with erythrosin B (100 μg/ml) in Buffer A at 37°C for 5 min, and the percentage of stained cytoplasts was estimated in a hemacytometer. After 20 min of permeabilization, more than 90% of the cytoplasts were stained. The hemoglobin content was determined as described elsewhere (17).

Labeling and Preparation of RNA

Cytoplasts were resuspended in fresh medium containing 2% Ficoll (2–5 × 10^7 cells per ml), supplemented with [5,6-^{3}H]uridine (60 Ci/mmol, Amersham Corp, Arlington Heights, IL) to 2 mCi/ml, and incubated at 37°C for appropriate time. Where indicated, actinomycin D (20 μg/ml) was added 5 min before addition of radioactivity.

Permeabilized cytoplasts were labeled with [^{3}H]uridine in a buffer containing 50 mM Hepes, pH 7.6, 80 mM NaCl, 20 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 2% Ficoll, 5 mM potassium phosphate, pH 7.6, 2.5 mM ATP, 0.2 mM CTP and GTP, 0.01 mM [^{3}H]UTP, 5 μg/ml polyvinylsulfate, and 100 μg/ml saponin at 30°C for 30 min. Labeling with [5-Hg]cytidine was done in a similar mixture containing 0.05 mM [5-Hg]CTP and 0.25 mM CTP.

Labeled cytoplasts or permeabilized cytoplasts were collected by centrifugation and lysed by addition of a buffer containing 5 mM NaCl, 3 mM $MgCl_2$, 30 mM Hepes, pH 7.8, and 1% Triton X-100. The lysates were repeatedly extracted with penol and chloroform-isoamyl alcohol until the interphase was clear, and the resulting RNA was precipitated with ethanol.

Assay of Protein Synthesis

Permeabilized cytoplasts were collected by centrifugation and incubated at final concentration of 2 × 10^7 cytoplasts per ml in buffer containing 100 mM KOAc, 5 mM MgOAc, 50 mM tricine, pH 7.4, 20 μM L-[^{3}H]leucine, 0.2 mM of the other 19 amino acids, 0.1 mM GTP, 1 mM ATP, 5 mM phosphoenolpyruvate, 0.2 unit pyruvate kinase, and 2 mM 2-mercaptoethanol at 30°C. At appropriate times of incubation, samples were removed and processed by trichloroacetic acid (TCA) precipitation as described elsewhere (20).

Fractionation of RNA

For size fractionation, RNA samples were suspended in 100 μl of 85% formamide containing 10 mM Tris, pH 7.6, and 1 mM EDTA and heated at 65°C for 5 min before loading on the top of a 2–10% sucrose gradient in 85% formamide, 10 mM Tris, pH 7.6, and 1 mM EDTA. The gradient was centrifuged at 49,000 rpm in a SW50.1 rotor (Beckman Instruments, Inc., Palo Alto, CA) for 14 h at 20°C.

Separation of RNA containing mercurated cytidine involved affinity chromatography on thiol-agarose (Affigel 401, Bio-Rad Laboratories, Richmond, CA) as described elsewhere (2). RNA was denatured by incubation at 70°C in 10 mM Tris, pH 7.4, 1 mM EDTA before application to thiol-agarose and the bound fraction was eluted with a buffer containing 2-mercaptoethanol and 10 μg/ml tRNA as a carrier. Less than 2% of RNA sample was bound to and eluted from thiol-agarose.

Hybridization of RNA with Globin DNA-Cellulose

RNA was hybridized to an excess of single-stranded recombinant M13 DNA containing the PstI–PstI fragment of mouse β maj globin gene in either of two orientations and covalently bound to cellulose (23). Each mixture contained an equivalent of at least 30 μg DNA and was incubated under the hybridization conditions described earlier (30). To ensure an excess of globin DNA cellulose, we determined a concentration curve for each preparation of globin DNA–cellulose with in vivo-labeled ^{3}H RNA as described elsewhere (1). To ensure that DNA was in excess, equal amounts of DNA–cellulose were hybridized with double amounts of labeled RNA in control hybridization.

All results were corrected for the background that was determined for each experiment by hybridizing equal amounts of radioactive RNA no nonrecombinant M13 DNA–cellulose and was 0.01% of RNA input or less.

Results

Preparation of Cytoplasts of Differentiated MEL Cells in the Absence of Cytochalasin B

Enucleation of cells in suspension is usually achieved by treatment with cytochalasin B (10 μg/ml) followed by isopycnic centrifugation in a discontinuous Ficoll gradient. As a result of the disruption of the cytoskeleton by cytochalasin B, the centrifugal force leads to the extrusion of the denser nuclei from the cell, yielding the less dense cytoplast fraction, i.e., enucleated cells, and the denser karyoplasts, nuclei surrounded by plasma membrane. In the course of our study we discovered, however, that differentiating MEL cells could be enucleated in the absence of cytochalasin B and that this susceptibility to enucleation in a density gradient is a function of differentiation. When mixed populations of differentiating and uninduced cells were subjected to density gradient centrifugation without treatment with cytochalasin B, undifferentiated cells remain intact and banded midway between cytoplasts and karyoplasts derived from differentiated cells. The integrity of cytoplasts of differentiating MEL cells obtained by such a procedure, as judged by morphology and rate of protein synthesis, was significantly greater than that of cytoplasts obtained with cytochalasin B (data not shown). The cytoplasts obtained in this way originated exclusively from differentiating cells and contained less than 0.1% of intact cells and karyoplasts as judged by Giemsa and Feulgen staining.

Synthesis of Positive and Negative Strands of Globin RNA in Cytoplasts of Differentiating MEL Cells

Cytoplasts were collected as the upper band in a Ficoll gradient as described above and were labeled for different periods with [^{3}H]uridine, lysed, and subjected to phenol

Table I. Synthesis of Positive and Negative Strands of Globin RNA in Enucleated Cells

Origin of enucleated cells	Labeling period	Actinomycin D added	Radioactivity in positive-strand globin RNA	Radioactivity in negative-strand globin RNA
	min	*μg/ml*	*cpm*	*cpm*
Differentiated MEL cells	15	0	261	112
Differentiated MEL cells	30	0	611	184
Differentiated MEL cells	30	20	563	167
Differentiated MEL cells*	30	0	412	141
Differentiated MEL cells	45	0	817	194
Differentiated MEL cells	60	0	931	181
Differentiated MEL cells	60	20	943	183
Undifferentiated MEL cells	30	0	34	0
Undifferentiated MEL cells	60	0	39	0
L cells	30	0	0	0
L cells	60	0	0	0

Enucleated cells (1×10^8) were labeled with [^{3}H]uridine as indicated. RNA was isolated and hybridized to an excess of single-stranded M13 DNA containing β maj globin DNA in the appropriate orientation and the amount of radioactive RNA hybridized was determined as described in the text.
* Labeled RNA was hybridized with PstI–XbaI fragment of globin DNA in the appropriate orientation. (PstI–PstI fragment corresponds to 367 5′-end nucleotides, PstI–XbaI fragment corresponds to 259 3′-end nucleotides.)

chloroform extraction. Isolated RNA was hybridized with two preparations of immobilized single-stranded M13 DNA containing globin DNA in the two possible orientations. RNA isolated from cytoplasts of L cells or of uninduced MEL cells, obtained using cytochalasin B, served as controls. As shown in Table I, the synthesis of both positive- and negative-globin RNA strands was observed in cytoplasts of differentiating MEL cells and was only slightly inhibited by actinomycin D. Whereas labeled globin mRNA accumulated continuously during labeling, the level of labeled RNA corresponding to the globin-negative strand reached a plateau within 30 min, suggesting that it is subject to a rapid turnover. It is unlikely that the appearance of negative-strand RNA was an artifact due to hybridization of labeled positive-strand to contaminating double-stranded recombinant M13 DNA, since DNA was immobilized under nondenaturing conditions where double-stranded DNA binds minimally. Moreover, double-stranded DNA, even if bound, would not effectively hybridize with labeled RNA under the conditions used. To confirm this conclusion, globin cDNA was cloned in pGEM (Promega Biotec, Madison, WI) vector containing SP6 and T7 promoters and radioactive positive- and negative-globin mRNA strands were synthesized from linearized DNA using appropriate RNA polymerases. These RNA preparations were hybridized with hybridization matrix containing single-stranded DNA complementary to positive or negative strands of globin RNA. Both hybridization matrixes hybridized exclusively with an appropriate RNA preparation. To test the possibility of cellular DNA interference with our measuring procedure, the identical experiments were carried out using RNA preparations pretreated with DNase A (Worthington Biochemical Corp., Freehold, NJ, RNase-free, 20 min, 20°C in 10 mM Tris, pH 7.5, 10 mg $MgCl_2$). The results were essentially the same as shown in Table I, ruling out this possibility.

Both labeled positive and negative strands of globin mRNA migrated as 9S RNA in formamide-sucrose gradient (Fig. 1).

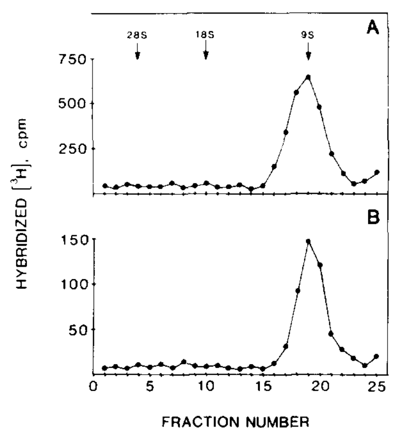

Figure 1. Sedimentation behavior of globin RNA labeled in cytoplasts of differentiated MEL cells. Cells were treated for 4 d with 4 mM hexamethylenebisacetamide and enucleated as described. 2×10^8 cytoplasts were labeled for 60 min with [^{3}H]uridine, total RNA was isolated, dissolved in 85% formamide containing 10 mM Tris, pH 7.6, and 1 mM EDTA, denatured by heating at 65°C and fractionated on sucrose-formamide gradient. Each of the gradient fractions was hybridized to an excess of single-stranded globin DNA in M13 covalently bound to cellulose. (*A*) Hybridization matrix contains single-stranded DNA complementary to the positive-strand globin RNA. (*B*) Hybridization matrix contains single-stranded DNA complementary to the negative-strand globin RNA.

Preparation and Characterization of Permeabilized Cytoplasts of Differentiating MEL Cells

Permeabilization of cytoplasts was achieved by treatment with saponin (24) and dextran sulfate (16). The following criteria were used to evaluate the procedure: (*a*) permeability to small molecules; (*b*) cellular integrity; and (*c*) ability to support synthesis of globin mRNA. Uptake of the vital dye erythrosin B, which is negatively charged and cannot cross the intact plasma membrane, was used as a measure of permeabilization. Another criterion of cell permeability was the partial release of hemoglobin after treatment. The ability to support synthesis of proteins as measured by incorporation of radioactive amino acids was used as an indication of cellular integrity. The ability to support synthesis of globin mRNA was evaluated by labeling permeabilized cytoplasts

with tritiated or [^{32}P]UTP, isolating RNA and assaying it by hybridization to immobilized recombinant M13 DNA. Treatment with a combination of dextran sulfate and saponin gave satisfactory permeabilization according to these criteria, leading to erythrosin B staining of more than 90% of the cytoplasts and the release of 30% of cellular hemoglobin, while the incorporation of tritiated leucine into protein was linear for 45 min and that of radioactive UTP into globin mRNA for at least 30 min.

Radioactivity Is Incorporated Only into Newly Synthesized Globin mRNA Molecules

A possible explanation for the observed cytoplasmic labeling of globin mRNA is the modification of preexisting globin RNA. The possibility of terminal addition of nucleotides to preexisting globin mRNA was addressed previously by using the cloned MboII–HindIII fragment of the β maj globin gene which contains the second of the three globin gene exons, as hybridization template, followed by treatment with RNase A (30). The observed hybridization with the internal exon indicated that cytoplasmic labeling of globin RNA was not simply due to end addition. The availability of permeabilized cytoplasts made possible another approach to the question of de novo synthesis of globin mRNA. This involved double labeling with [^{3}H]UTP and [5-Hg]CTP and took advantage of the fact that nucleic acids containing mercurated nucleotides bind specifically to thiol-substituted agarose. This binding requires about one mercury derivative in 100–200 nucleotides (2, 7) or ∼3–6 Hg for a molecule the size of 9S globin mRNA. Under our experimental conditions, which involved the use of CTP and [5-Hg]CTP in a 5:1 molar ratio, the incorporation of at least 18–36 cytidylate residues or ∼70–140 nucleotides would thus be necessary for the binding of globin mRNA to thiol-agarose. The determination of the relative degrees of [^{3}H]UMP incorporation into globin mRNA fractions bound or unbound by thiol-agarose would therefore provide an indication whether the ^{3}H-labeled molecules were the products of limited or extensive synthesis. The results of such an experiment are shown in Table II and indicate that radioactivity was exclusively incorporated into the globin mRNA fraction that bound to thiol-agarose, although that fraction presumably represents only a very small proportion of the total globin mRNA. It appears, therefore, that cytoplasmic labeling of globin mRNA represents relatively extensive, i.e., de novo, synthesis and not the addition of just a few nucleotide residues at the ends of the globin mRNA molecules.

It was suggested by one of the reviewers that these results are also consistent with the possibility that small fragments of both plus and minus sense of globin RNA (which may be generated by natural degradation of globin RNA or by its degradation during preparation of permeabilized cytoplasts) may be used as primers on appropriate template RNA for synthesis of new RNA products. If this is the case, one may expect that position of initiation and thus the 5′ end of newly synthesized RNA will be determined by the priming fragment. On the other hand, the 3′ end of the newly synthesized RNA will correspond to the 5′ end of the template. To examine this possibility, labeled RNA was hybridized with an excess of single-stranded recombinant M13 DNA containing only the 3′ part (PstI–XbaI fragment) of the globin gene in either of the two orientations and covalently bound to cellulose (30). With random initiation and constant termination site, one would expect that more newly synthesized RNA will hybridize with the 5′ end fragment of the gene than the 3′ end fragment (at least for minus sense RNA). However (Table I) proportionally equal amounts of newly synthesized RNA hybridize with both end fragments. Although these results do not exclude the possibility that 3′ end fragments are preferentially used to prime the synthesis of complementary RNA strand, taken together with the results presented in Fig. 1 they strongly suggest synthesis of a full-size RNA copy.

The permeabilized cytoplast system also allowed us to address in more detail the possibility of DNA involvement. One may assume that permeabilized cells or permeabilized cytoplasts depend entirely on added nucleotide triphosphates (NTPs) or deoxynucleotide triphosphates (dNTPs) for RNA or DNA synthesis. This is definitely true in our case – if only one labeled NTP is added to permeabilized cells or cytoplasts or if only one labeled dNTP is added to permeabilized cells, no incorporation is observed. Since no dNTPs were added to enucleated cytoplasts, we assume that no DNA synthesis is involved in the observed phenomenon. Moreover, the addition of 2 mM ddATP in our experiment does not change its outcome.

Table II. Incorporation of [5-Hg]CTP and [^{3}H]UTP into Positive and Negative Strand Globin RNA in Permeabilized Cytoplasts of Differentiated MEL Cells

Fraction of RNA	Radioactivity in positive-strand globin RNA	Radioactivity in negative-strand globin RNA
	cpm	*cpm*
Bound to thiol-agarose	932	185
Not bound to thiol-agarose	51	16

Enucleated cells (3 × 10^{8}) were permeabilized and labeled with [5-Hg]CTP and [^{3}H]UTP for 30 min, RNA was isolated, separated on thiol-agarose into a mercurated (bound) and an unmercurated (unbound) fraction, and hybridized to an excess of single-stranded M13 DNA containing β maj globin DNA in appropriate orientation, and the amount of radioactivity hybridized in each fraction was determined. (For details, see text.)

Divalent Cation Requirements for Synthesis of Globin RNA in Permeabilized Cytoplasts

The availability of permeabilized cytoplasts also allowed us to compare certain properties of cytoplasmic RNA synthesis with those of known nucleic acid polymerases. It was shown previously that Mg^{++} is essential for the activity of RNA-dependent RNA polymerases in viral/eukaryotic systems and cannot be replaced by Mn^{++}, which strongly inhibits the enzyme activity (11, 21). The optimum Mg^{++} concentration for the picornaviral RNA replicases was very narrow (8). The rate of cytoplasmic globin RNA synthesis showed little variation with Mg^{++} in the 5–20 mM range (data not shown). However in the absence of Mg^{++}, both positive- and negative-strand synthesis of globin RNA was reduced almost 10-fold (Table III). Replacement of Mg^{++} with Mn^{++} restored the activity to only 25% of the control level, and Mn^{++} inhibited the activity in the presence of Mg^{++}. Another divalent cation, Zn^{++}, essential for all DNA and RNA polymerases examined (3, 19), can replace Mg^{++} with DNA polymerases from various organisms (27). In our system,

Table III. Divalent Cation Requirement for Synthesis of Globin RNA in Permeabilized Cytoplasts of Differentiated MEL Cells

Divalent cations added	[^{3}H]UMP incorporated into positive-strand globin RNA	[^{3}H]UMP incorporated into negative-strand globin RNA
	cpm	*cpm*
None	124	28
10 mM $MgCl_2$	1071	198
1 mM $MnCl_2$	187	54
10 mM $MnCl_2$	272	61
10 mM $MgCl_2$, 10 mM $MnCl_2$	294	78

Enucleated cells (3 × 10^8) from 4-d-induced MEL cells were permeabilized and labeled with [^{3}H]UTP for 30 min in a mixture containing the indicated levels of divalent cations. RNA was isolated, hybridized with an excess of single-stranded M13 DNA containing β maj globin DNA in appropriate orientation, and the amount of radioactivity hybridized was determined.

however, the addition of zinc in a wide range of concentrations (10–100 mM) had no significant effect on the synthesis of globin RNA, nor could it replace Mg^{++} (data not shown).

Effect of Inhibitors of Nucleic Acid Synthesis

The response of the RNA synthesizing activity in our system to certain inhibitors of nucleic acid synthesis was also examined (Table IV). In agreement with our previous observations in whole cells (30) and intact cytoplasts (Table I) globin RNA synthesis was not inhibited by actinomycin D. It also was resistant to α-amanitin and to rifampicin, but was inhibited, by the rifamycin derivative AF/ABDP and by aurintricarboxylic acid.

Discussion

Earlier experiments had suggested that globin mRNA synthesis could occur in the cytoplasm of differentiating MEL cells, presumably by a mechanism that involved RNA-dependent RNA synthesis and RNA complementary to globin mRNA ("negative-strand" globin RNA) as an intermediate (30). The evidence for this conclusion came in part from studies of the kinetics of globin RNA synthesis, its response to inhibitors, and the localization of the product, none of which alone constituted definitive proof for our hypothesis.

To demonstrate directly the occurrence of cytoplasmic synthesis of globin mRNA, we turned to the study of enucleated cells. Our approach was based on the earlier observation by Prescott et al. (26) that cultured mammalian cells could be readily enucleated after treatment with cytochalasin B by exposure to a centrifugal field. This procedure originally involved centrifugation of cells attached to a surface but was subsequently extended by Wigler and Weinstein (34) to cells in suspension by the use of isopycnic centrifugation in a discontinuous density gradient of Ficoll 400 containing cytochalasin B. Preparations of cytoplasts and karyoplasts obtained in this manner have been used in various investigations, such as studies on the site of replication of a variety of viruses (15, 28, 29), the cellular responses to hormones and interferon (14, 36), and localization of various cellular functions (5, 6). Centrifugal field-induced enucleation could also be achieved with MEL. However, in the course of studying this process we made the very useful observation that differentiating MEL cells were enucleated upon isopycnic centrifugation even in the absence of cytochalasin B, perhaps due to a cytoskeleton alteration accompanying the differentiation process. Cytoplasts produced in the absence of cytochalasin B were not only obtained with better yields, but showed a higher degree of functional integrity as indicated by the rate of [^{3}H]leucine incorporation into protein (our unpublished data). Another advantage accrued from the fact that only differentiating MEL cells were enucleated in the absence of cytochalasin B while undifferentiating cells remained intact and banded at a different position in the Ficoll density gradient. Accordingly, even with mixed cell populations, cytoplasts from differentiating cells could be obtained 99.9% pure from the uppermost band in the density gradient.

Table IV. Effect of Inhibitors on Positive and Negative Strand Globin RNA Synthesis in Permeabilized Cytoplasts of Differentiated MEL Cells

Inhibitor added	[^{3}H]UMP incorporated into positive-strand globin RNA	Inhibition	[^{3}H]UMP incorporated into negative-strand globin RNA	Inhibition
	cpm	%	*cpm*	%
None	1071	–	198	–
Actincomycin D, 20 mg/ml	952	11	204	0
α-Amanitin, 20 μg/ml	912	14	167	15
Rifampicin, 100 μg/ml	906	15	174	12
Rifamycin AF/ABDP, 100 μg/ml	331	69	48	78
Aurincricarboxylic acid, 5 μM	217	79	22	82

Enucleated cells (3 × 10^8) from 4-d-induced MEL cells were permeabilized and labeled with [^{3}H]UTP for 30 min in the presence of inhibitors as indicated. RNA was isolated, hybridized with an excess of single-stranded M13 DNA containing globin DNA in appropriate orientation, and the amount of radioactivity hybridized was determined.

The availability of cytoplast preparations from differentiating MEL cells, completely free of contaminating nucleated cells, allowed us to establish definitively the occurrence of the cytoplasmic synthesis of both positive- and negative-strand globin mRNA. Studies of the kinetics of this process indicated that the accumulation of negative-strand globin RNA reached a maximum while positive-strand accumulation continued, consistent with the notion that the negative-strand RNA is a relatively unstable intermediate template in the RNA-dependent synthesis of globin mRNA. The rate of labeling of globin mRNA in cytoplasts was 30–50% of that seen earlier with whole differentiating MEL cells (30).

To characterize further the properties of cytoplasmic globin RNA synthesis, we subjected cytoplasts to selective permeabilization by treatment with saponin and dextran sulfate. This allowed the study of the effect of substances that do not readily cross the plasma membrane, such as nucleotide substrates, metal ions, and various inhibitors. By using mercury-substituted CTP as substrate, it was possible to separate newly synthesized globin RNA from bulk globin mRNA by adsorption to thiol-agarose and show that the cytoplasmic incorporation of radioactive precursors into both positive- and negative-strand globin RNA involved substantial synthesis of RNA molecules rather than the terminal addition of a few nucleotide residues to preexisting RNAs. It was also possible to examine the divalent cation requirement of cytoplasmic globin RNA synthesis and show that it depended on the presence of Mg^{++}, was inhibited by Mn^{++}, and showed no response to Zn^{++}. These properties can be compared to those of viral RNA replicases, which are also Mg^{++}-dependent and inhibited by Mn^{++} but are stimulated by the addition of Zn^{++} (3, 8). The lack of effect of Zn^{++} in our system could be due to the presence of saturating levels of this ion in the cytoplast preparations, and studies with specific chelators will be necessary to determine whether cytoplasmic globin RNA synthesis is indeed Zn^{++}-dependent as all other DNA and RNA polymerases described to date (3, 14, 27).

We also tested the effect of several inhibitors of RNA synthesis on the synthesis of globin RNA in our assay system. We found it to be resistant to actinomycin D and α-amanitin, both potent inhibitors of DNA-dependent RNA synthesis, as well as to rifampicin, an inhibitor of both bacterial and mitochondrial DNA-dependent RNA polymerases. The possibility that these inhibitors were not effective because they did not penetrate cytoplasts is unlikely in view of the fact that hemoglobin could pass through the plasma membrane of the permeabilized cytoplasts. Cytoplasmic globin RNA synthesis was inhibited by the rifamycin derivative AF/ABDP and by aurintricarboxylic acid. The former has been shown to inhibit a number of eukaryotic DNA-dependent DNA and RNA polymerases as well as RNA-dependent RNA polymerase (9, 35) and thus appears to be a general inhibitor of both DNA and RNA synthesis. Aurintricarboxilic acid has been shown to inhibit RNA-dependent DNA polymerase, as well as RNA-dependent RNA polymerases (4, 12) by binding to the template binding site on the polymerase. The obtained results strongly suggest that at least de novo DNA synthesis is not involved in the observed phenomenon: synthesis of RNA occurs in the permeabilized cytoplasts not only without addition of dNTPs but also when ddNTP is added.

MEL cells are known to harbor the Friend virus complex, which consists of a replication-defective murine leukemia virus and a helper virus, spleen focus-forming virus. Replication of these viruses occurs through a reverse transcription mechanism: viral RNA is used as a template by the virus-encoded enzyme, reverse transcriptase, to produce viral DNA. Since this mechanism operates in the cytoplasm, one may speculate that it might be involved in the observed phenomenon of cytoplasmic synthesis of globin mRNA in differentiating MEL cells. Several arguments, however, speak against this possibility. If reverse transcription were involved in the observed phenomenon, one would expect to find newly synthesized globin DNA. No such synthesis was observed in our experiments. One could speculate that a short-lived or preexisting DNA, synthesized by reverse transcriptase, might act as a template for the cytoplasmic synthesis of globin RNA. However, in that case, globin RNA synthesis, like all DNA-dependent RNA synthesis, should be sensitive to actinomycin D, contrary to our observations. Moreover, this mechanism could not account for the appearance of negative-strand globin RNA, which is specifically predicted by our model of RNA-dependent RNA synthesis. The detection of negative-strand globin RNA is a key discovery which strongly suggests that it and not a cDNA is an intermediate in the cytoplasmic synthesis of globin RNA. Finally, RNA-dependent RNA synthesis increases greatly during differentiation of MEL cells under conditions when reverse transcriptase activity rapidly declines, suggesting that these activities are unrelated.

The question remains, however, whether the observed mechanism indeed plays a physiological role in normal cells. Our recent results answer affirmatively to this question. Using spleen cells of anemic mice (number of different strands), we were able to reproduce all our findings with MEL cells. Moreover, we demonstrated that globin RNA synthesis also occurs in mature mouse reticulocytes (Volloch, V., B. Schweitzer, and S. Rits, manuscript in preparation).

The results of this study, combined with the results reported previously (30) provide strong support for our hypothesis that RNA-dependent RNA synthesis occurs in the cytoplasm of differentiating MEL cells. The mechanism and features of this synthesis as well as its biological significance and range of application are the subject of our current and future investigations, which take advantage of permeabilized enucleated cells as a convenient in vitro assay system.

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