Proc. Natl. Acad. Sci. USA
Vol. 74, No. 11, pp. 5011–5015, November 1977
Cell Biology

Definition of subclasses of nucleoplasmic RNA

(nuclear RNA-synthesizing system/HeLa cells/5,6-dichloro-1-β-D-ribofuranosylbenzimidazole/α-amanitin/heparin)

IGOR TAMM

The Rockefeller University, New York, New York 10021

Contributed by Igor Tamm, August 9, 1977

ABSTRACT RNA synthesis in isolated HeLa cell nuclei prepared from cells pretreated with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) is inhibited in a time-dependent manner. After 40-min pretreatment of cells with 60 μM DRB in the presence of actinomycin D (0.04 μg/ml), the rate of RNA synthesis in isolated nuclei, measured by [^{3}H]UTP incorporation, is decreased by 63%. The DRB-resistant one-third of heterogeneous nuclear is distributed over the entire size range of heterogeneous nuclear RNA with some enrichment in the 18S range, as was observed earlier by pulse-labeling whole cells. A subclass of nucleoplasmic RNA molecules is defined in the approximate size range 110 to 250 $\times$ 10^3 daltons (330–740 nucleotides). By using heparin (2 mg/ml) to block the synthesis of smaller RNA, a peak in the chain-length range 330–740 nucleotides can be clearly resolved on 2.2% polyacrylamide/1% agarose gels in nuclei from control and DRB-treated cells. The synthesis of these molecules is largely (~90%) resistant to DRB but sensitive to α-amanitin at 1 μg/ml. The *in vitro* synthesis of molecules in the 140–330 residue range is also sensitive to α-amanitin at 1 μg/ml, and it is not at all affected by pretreatment of cells with DRB. Although the synthesis of the RNA in both the 330–740 and the 140–330 residue size ranges appears to be catalyzed by RNA polymerase II, the results with heparin suggest that there may be reinitiation of molecules in the 140–330 size range but not in the 330–740 range *in vitro*. The synthesis of 4.5S RNA (~100 nucleotides) and 5S RNA (120 nucleotides) is unaffected by pretreatment of cells with DRB and, as previously reported, is catalyzed by RNA polymerase III, with reinitiation occurring *in vitro*. Addition of DRB directly to isolated HeLa cell nuclei *in vitro* has no detectable effect on the overall rate of RNA synthesis.

5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) has a selective and reversible action on nuclear RNA synthesis in mammalian cells by inhibiting two-thirds of heterogeneous nuclear RNA (hnRNA) (1, 2) and >95% of mRNA (2) through an apparent block in chain initiation (3, 4).

Subclasses of DRB-resistant nucleoplasmic RNA have now been defined. The approach was to treat HeLa cells with DRB *in vivo* and then to analyze, on polyacrylamide gels, the RNA synthesized in isolated nuclei *in vitro* in the presence or absence of α-amanitin or heparin. The outstanding new result is the definition of a subclass of nucleoplasmic DRB-resistant (~90%) RNA in the approximate size range of 110–250 $\times$ 10^3 daltons (330–740 nucleotides). This RNA can be clearly resolved in nuclei from control and DRB-treated cells, in which the synthesis of smaller species has been blocked with heparin. The synthesis of molecules in the 330–740 residue size range appears to be catalyzed by RNA polymerase II and not to be reinitiated *in vitro*. The possible functions of these and other DRB-resistant molecules are considered.

MATERIALS AND METHODS

Cell Culture. HeLa S3 human carcinoma cells were grown and maintained in suspension culture in the spinner modification of Eagle's minimal essential medium (5) containing 5% fetal calf serum.

Chemicals and Buffers. [^{3}H]Uridine (21.0–29.65 Ci/mmol) and [^{3}H]uridine 5′-triphosphate tetrasodium salt ([^{3}H]UTP) (25.2 Ci/mmol) were obtained from New England Nuclear, Boston, MA. DRB (molecular weight 319.15) and actinomycin D were obtained from Merck Sharp & Dohme Research Laboratories (Rahway, NJ) through the courtesy of Arthur F. Wagner. DRB was dissolved in serum-free medium by shaking for several hours at 37°. α-Amanitin was a gift from Th. Wieland, Max-Planck Institut für Medizinische Forschung, Heidelberg, Germany. Heparin was purchased from Connaught Laboratories Ltd., ON, Canada, and Cleland's reagent (dithiothreitol) from Calbiochem, LaJolla, CA. ATP, CTP, GTP, and UTP were obtained from P. L. Biochemicals, Inc., Milwaukee, WI. The phosphate-buffered saline (PBS) was 0.13 M NaCl/2.7 mM KCl/0.82 mM Na_2HPO_4/1.5 mM KH_2PO_4/0.91 mM $CaCl_2$/0.5 mM $MgCl_2$; the pH was 7.3. The lysis buffer was 6 mM KCl/5 mM $MgCl_2$/0.01 M Tris, pH 7.4; to the buffer was added dithiothreitol to a concentration of 1 mM prior to use. High salt buffer was 0.5 M NaCl, 0.05 M $MgCl_2$, and 0.01 M Tris. Sodium dodecyl sulfate buffer ($NaDodSO_4$ buffer) was 0.5% $NaDodSO_4$/0.1 M NaCl/1 mM EDTA/0.01 M Tris, pH 7.4. Dialysis buffer was 0.01 M Tris, pH 7.4/0.1 M NaCl/0.5% $NaDodSO_4$.

The incubation mix for RNA synthesis in isolated nuclei contained 6 mM KCl, 1.6 mM $MnCl_2$, 0.1 M $(NH_4)_2SO_4$, 40 μM each of ATP, CTP, and GTP, 4 μM UTP, 2 μM [^{3}H]UTP (20–25 μCi per 500-μl sample), and 1 mM dithiothreitol in 50 mM Tris/25% glycerol, pH 7.6.

Isolation of Cell Nuclei and Nucleoplasmic and Nucleolar Fractions. The procedures used have been described (1, 6). Pertinent details are given in figure legends.

Pulse-Labeling Techniques. Isolated HeLa cell nuclei were labeled with [^{3}H]UTP for 0–30 min at 26°, and 25-μl aliquots were placed on Whatman 3 MM filters that were processed in batches. The acid-precipitable counts were expressed per total sample after subtraction of 0-min counts. RNA was extracted with phenol/chloroform (1). In most experiments, actinomycin D (0.04 μg/ml) was used to suppress ribosomal RNA synthesis.

Gel Electrophoresis. The procedure used has been described (1). The marks above the horizontal bar in each panel of Figs. 2, 3 and 4 refer, from left to right, to 28, 18, and 4S marker RNAs from HeLa cell cytoplasm. To obtain size estimates of RNA in the 4–18S range, the logarithms of the molecular weights for marker RNAs were plotted against migration rates, with molecular weights of 1,750,000, 605,000 and 25,000 for 28, 18, and 4S RNA, respectively (7). This gave a linear rela-

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; hnRNA, nuclear heterogeneous RNA; [^{3}H]UTP, [^{3}H]uridine 5′-triphosphate; PBS, phosphate-buffered saline; $NaDodSO_4$ buffer, sodium dodecyl sulfate buffer; NP, nucleoplasmic fraction; pre-4S RNA, ~4.5S precursor of tRNA.

Hunan University (222.247.53.42) - 2013/6/13 Download

tionship, which was used in interpolating molecular weights of RNAs in different gel fractions. The two marks below each bar are interpolated positions for RNA of 250,000 daltons (740 nucleotides) and 110,000 daltons (330 nucleotides).

RESULTS

Inhibition of RNA Synthesis in Isolated Nuclei after Pretreatment of Cells with DRB. Fig. 1*A* shows that pretreatment of HeLa cells with 60 μM DRB inhibits RNA synthesis in isolated nuclei, measured by incorporation of [^{3}H]UTP, and that the extent of inhibition is dependent on the duration of treatment of cells with DRB. Treatment for 2 min reduced the rate of RNA synthesis in isolated nuclei by approximately 14%, treatment for 10 min by 41%, and for 40 min by 63%. Closely similar levels of inhibition were observed when the *in vitro* reaction was carried out for 5, 15, or 30 min (Fig. 1*B*). The fact that the major part of the inhibitory effect on RNA synthesis in isolated nuclei was established after 10 min of treatment of whole cells with 60 μM DRB agrees with previous results obtained by pulse-labeling whole cells (2).

When added directly to isolated nuclei, DRB had no detectable effect on the overall rate of RNA synthesis.

Size Distribution of RNA Made *In Vitro* in Nuclei Isolated from DRB-Treated Cells. HeLa cells were treated with 60 μM DRB for 10 or 40 min. Nuclei were then isolated and incubated *in vitro* with nucleoside triphosphates, including [^{3}H]UTP, for 15 min. Fig. 2 shows the results of electrophoretic analysis, on 1.1% polyacrylamide/1% agarose gels, of RNA isolated from the nuclei. RNA from controls shows that the major distribution of labeled molecules is in the >18S range. Because denaturing conditions were not used, the exact size distribution of hnRNA chains cannot be inferred (7, 8). In the controls, there was also present a second minor peak in the size range of $\sim$33–250 $\times$ 10^3 daltons (100–740 nucleotides). The profile of RNA made in isolated nuclei after treatment of cells with DRB for 10 min (Fig. 2*A*) shows considerable depression over the entire hnRNA range but little change in the labeling of RNA in the 100–740 nucleotide range. After treatment of cells with DRB for 40 min (Fig. 2*B*), the predominant peak of RNA labeled *in vitro* was in the 100–740 nucleotide range, which now overshadowed the broad distribution of DRB-resistant one-third of hnRNA. Synthesis of this DRB-resistant RNA in the nucleoplasm (NP) of cells that had not been treated with actinomycin D has also been observed (data not shown).

The following procedure was used to determine the effect of DRB on the synthesis of RNA in different size categories. The dpm recovered in groups of gel fractions was calculated as a percentage of total dpm recovered from the entire gel and then this percentage was applied to the total trichloroacetic acid-precipitable dpm in the sample before phenol extraction. The mean results of three experiments, summarized in Table 1, are in close agreement with the results shown in Fig. 2. The quantitative analysis reveals several features. RNA synthesis in the size range of 910–2400 nucleotides (including 18S molecules) is somewhat less sensitive to inhibition by DRB than that in the range of the largest hnRNA molecules (2). Synthesis of RNA in the 330–740 nucleotide range shows only 27% sensitivity and it is likely that a part of this apparent sensitivity is due to the presence of a shoulder of more sensitive hnRNA molecules in this region of the gel (see below). RNA <330 residues long is completely resistant to inhibition by DRB (Table 1). This includes $\sim$4.5S precursor of tRNA (pre-4S RNA) ($\sim$100 nucleotides) and 5S RNA (120 nucleotides) (data not shown).

It was determined that RNA molecules in the 100–740 nucleotide range do not represent degradation or processing products derived from longer chains (data not shown).

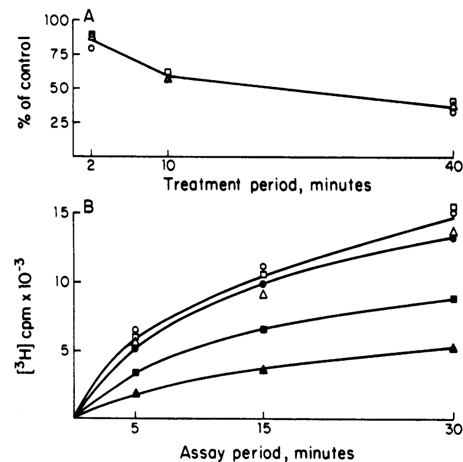

FIG. 1. Effect of pretreatment of HeLa cells with DRB on RNA synthesis in isolated nuclei. Cells were collected by centrifugation when the density in suspension cultures reached 2 $\times$ 10^5 cells per ml. They were washed once with warm spinner medium and resuspended at 1 $\times$ 10^6 cells per ml in spinner medium containing 5% fetal calf serum and actinomycin D at 0.04 μg/ml. The cells (10^7) in 10-ml aliquots were incubated for 25 min at 37° in a water bath. Each aliquot was then divided into two 5-ml portions to which was added an equal volume of serum- and actinomycin D-containing spinner medium or the same medium but also containing 120 μM DRB. The samples were incubated for 2, 10, or 40 min at 37°, after which the cells were harvested by pouring onto crushed frozen PBS and centrifugation. They were washed once with PBS and suspended for 15 min in lysis buffer (1 ml) containing 1 mM dithiothreitol on ice. The cells were then broken with 20–30 strokes in a tight-fitting Dounce homogenizer. The nuclei were collected by centrifugation and washed once or twice with lysis buffer; then the nuclear pellet was drained. The nuclei were then suspended in 100 μl of Tris/glycerol buffer and the incubation mix for assay of RNA synthesis (including [^{3}H]UTP, 10 μCi per sample) was added to a total volume of 250 μl. Actinomycin D (0.04 μg/ml) was present during the assay. The nuclear preparations were incubated at 26°. At 0, 5, 15, and 30 min, acid-precipitable counts were determined. The mean results of six experiments are shown. (*A*) Relationship between duration of treatment of cells with DRB and the rate of RNA synthesis in isolated nuclei, expressed as % of control. Assay periods: ○, 5 min; □, 15 min; △, 30-min. (*B*) Kinetics of [^{3}H]UTP incorporation by isolated nuclei from control cells (○, □, △) or DRB-treated cells (●, ■, ▲) incubated for 2, 10, or 40 min, respectively.

The question posed by the finding of DRB-resistant RNA as a major peak in the 100–740 nucleotide range is whether subclasses of molecules can be distinguished on a functional basis within this range.

Three Groups of Nuclear RNA in the 100–740 Nucleotide Range. α-Amanitin and heparin were used to distinguish among subclasses of DRB-resistant RNA in the 100–740 nucleotide range. α-Amanitin at low concentrations inhibits RNA chain growth catalyzed by RNA polymerase II, the enzyme responsible for the synthesis of hnRNA and mRNA (reviewed in ref. 9). At a concentration of 0.5 μg/ml it has also been shown to inhibit the synthesis of $\sim$5.3–6.6S but not that of 4.5–5S RNA in isolated HeLa cell nuclei *in vitro* (10). At 150 μg/ml, α-amanitin does inhibit RNA polymerase III, which catalyzes the synthesis of pre-4S and 5S RNA in isolated nuclei (11–13).

Fig. 3 *A–C* shows results that are entirely in accord with the earlier observations cited above. In addition, it is evident in Fig. 3*B* that the synthesis of RNA in the 330–740 nucleotide range is sensitive to α-amanitin at the concentration of 1 μg/ml. This result was confirmed in repeated experiments using higher concentration gels. Use of such gels has also shown that the synthesis of RNA >5S (>120 nucleotides) is largely sensitive at 1 μg/ml and that the synthesis of 5S and smaller RNA is in-

Hunan University (222.247.53.42) - 2013/6/13 Download

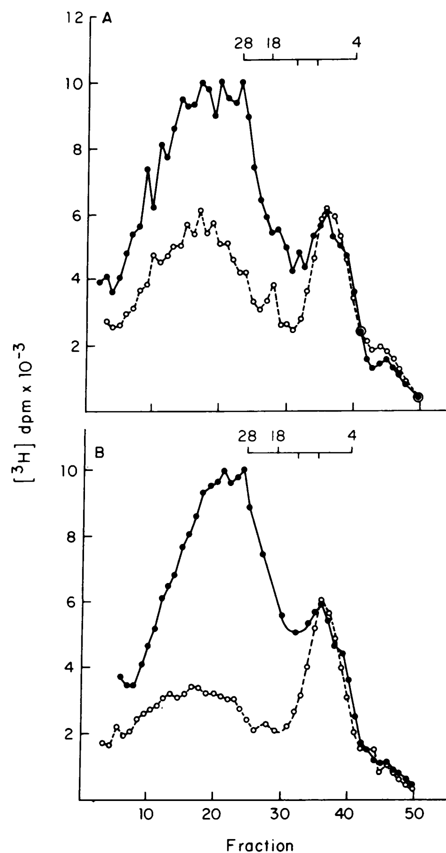

FIG. 2. Effect of pretreatment of HeLa cells with DRB on the synthesis of (hnRNA) and 100–770 nucleotide RNA in isolated nuclei. Cells were prepared as described in Fig. 1 and treated with 60 μM DRB for 10 or 40 min. Duplicate samples (10^7 cells each) were used per variable. The nuclei were isolated and RNA synthesis was assayed as described in Fig. 1. The 15-min acid-precipitable counts were as follows: 10-min control, 7.93×10^4 cpm; 10-min DRB, 4.88×10^4 (62%); 40-min control, 8.04×10^4; 40-min DRB, 3.87×10^4 (48%). The remainder of each sample was suspended in 2 ml of high-salt buffer and treated with DNase (12.5 μg/ml) for 15 min at room temperature. The nucleoplasmic (NP) and nucleolar (NL) fractions were separated by zonal centrifugation. The nuclear digest was layered on 2 ml of 30% sucrose in HSB and centrifuged at 23,000 rpm for 10 min in an SW 56 Beckman rotor. The NP components in the supernatant were precipitated with 2 volumes of ethanol, collected by centrifugation, and resuspended in 1 ml of $NaDodSO_4$ buffer. The acid-precipitable counts were as follows: 10-min control, 7.49×10^4 cpm; 10-min DRB, 5.30×10^4 (71%); 40-min control, 9.17×10^4; 40-min DRB, 3.85×10^4 (42%).

The remaining portions of the duplicate samples of NP material were pooled and cytoplasmic [^{14}C]RNA marker (50,000 cpm equivalents) was added to each pool. The material was brought to a final concentration of 0.01 M EDTA, and RNA was extracted with phenol/chloroform (1). The RNA preparations were dialyzed overnight against Tris/NaCl/$NaDodSO_4$ and precipitated with ethanol at −20 ° for 2 hr. The precipitates were resuspended in 100 μl of electrophoresis buffer and analyzed by gel electrophoresis in 1.1% polyacrylamide/1% agarose gels (1). Recoveries of label from control and experimental gels were comparable. (*A*) ●—●, Control; ○ - - - ○, 60 μM DRB, 10 min (*in vivo*). (*B*) ●—●, Control; ○ - - - ○, 60 μM DRB, 40 min (*in vivo*).

hibited by α-amanitin at 150 μg/ml. As reported previously (10–12), processing of pre-4S RNA to 4S RNA did not occur in isolated nuclei *in vitro* (data not shown).

Heparin, 0.5–2.0 mg/ml, inhibits the initiation of RNA chains (14) in isolated nuclei *in vitro* (13, 15). Heparin stimulates the synthesis of already initiated chains, possibly because of a change in chromatin structure or inhibition of RNase activity (13, 14). Addition of heparin, to 1 or 3 mg/ml, causes swelling

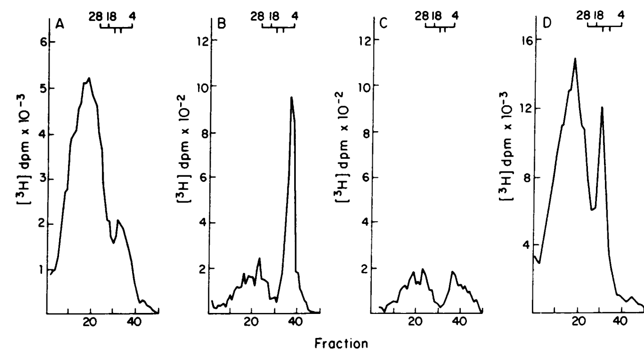

FIG. 3. Effects of α-amanitin and heparin on the synthesis of hnRNA and 100–770 nucleotide RNA in isolated HeLa cell nuclei. Cells were prepared and processed as described in Figs. 1 and 2, except that no actinomycin D or DRB was used. Labeling of nuclei with [^{3}H]UTP was carried out for 30 min in a volume of 0.5 ml containing nuclei from 2×10^7 cells. NP samples were used for isolation of RNA that was analyzed by electrophoresis on 1.1% polyacrylamide/1% agarose gels (1). (*A*) Control. (*B*) α-Amanitin, 1 μg/ml. (*C*) α-Amanitin, 150 μg/ml. (*D*) Heparin, 2 mg/ml.

and some lysis of isolated mouse cell nuclei (16). Heparin alters nuclear ultrastructure and displaces some nuclear proteins (17). In the present experiments, heparin at 2 mg/ml disrupted HeLa cell nuclei. The fact that heparin inhibits RNase activity is probably not relevant to the system of isolated HeLa cell nuclei because there appears to be very little RNase activity associated with such nuclei (13).

It has been shown that RNA polymerase III is capable of repeatedly initiating new chains of RNA in isolated nuclei *in vitro* (12, 13, 18). It would therefore be expected that treatment of isolated nuclei with heparin would largely eliminate the synthesis of RNA in the 4–5S range. Fig. 3*D* suggests that heparin does block the synthesis of such RNA; however, this is difficult to quantitate because of a stimulation of synthesis in the chain length range of 300–1000 nucleotides. There is also a considerable increase in synthesis over the entire range of hnRNA in the presence of heparin.

Use of 2.2% polyacrylamide/1% agarose gels provides better resolution of the DRB-resistant RNA in the 330–740 nucleotide size range as well as of larger RNA, whose synthesis *in vitro* is inhibited by α-amanitin but not by heparin. As illustrated in Fig. 4, treatment with heparin, by largely eliminating the synthesis of RNA chains shorter than about 300 nucleotides, makes possible the clear resolution of a peak of RNA in the size range of 110–250 × 10^3 daltons or 330–740 nucleotides. This RNA is largely resistant to inhibition by DRB. After correction for a slight difference in recovery, the radioactivity in the 110 to 250 × 10^3 dalton peak in the DRB-treated sample is equivalent to 90% of that in the control.

The RNA profiles in Fig. 4 suggest that heparin blocks not only pre-4S (~100 nucleotides) and 5S (120 nucleotides) RNA synthesis but also synthesis of chains in an intermediate size range, between 120 and ~300 nucleotides. Because RNA synthesis in this range is largely sensitive to α-amanitin at 1 μg/ml, the possibility arises that RNA polymerase II may be reinitiating the synthesis of such chains in isolated HeLa cell nuclei *in vitro*.

Fig. 4*B* shows that there is RNA in the 18S size range, whose resistance to DRB is intermediate between the lesser resistance of larger hnRNA and the greater resistance of RNA in the 330–740 nucleotide range (cf. ref. 2 and Table 1).

DISCUSSION

The outstanding characteristic of DRB is its selective inhibitory effect on the synthesis of two-thirds of nuclear hnRNA and

Hunan University (222.247.53.42) - 2013/6/13 Download

Table 1. Synthesis of RNA in isolated HeLa cell nuclei after treatment of cells with 60 μM DRB for 40 min

Approximate RNA size in groups of gel fractions		Total gel dpm*: Control 196,629, DRB 71,654 — Gel dpm		% of total gel dpm		Total acid dpm†: Control 418,794, DRB 195,797 — Estimated acid dpm†		DRB, % of control
Daltons × 10^{-3}	Nucleotides, no.	Control	DRB	Control	DRB	Control	DRB	
1,000–10,000‡	3,000–30,000	57,094	16,516	29	23	121,436	45,070	37
300–800	910–2,400	14,630	5,119	7.4	7.1	30,947	13,861	45
110–250	330–740	15,358	8,701	7.8	12	32,691	23,712	73
47–90	140–270	12,314	10,047	6.3	14	26,414	27,373	104

*1.1% polyacrylamide/1% agarose gels.

† Trichloroacetic acid-precipitable.

‡ >10 × 10^6 dalton RNA was not included, although calculations showed that its sensitivity to inhibition by DRB was similar to that of 1 to 10 × 10^6 dalton RNA.

>95% of mRNA in mammalian cells (1, 2). We have now found, by pretreating HeLa cells with DRB and measuring RNA synthesis in isolated nuclei, that the synthesis of RNA in the approximate size range of 33–250 × 10^3 daltons (100–770 nucleotides) is largely resistant to inhibition by DRB. The resistance of pre-4S RNA (~100 nucleotides) and 5S RNA (120 nucleotides) is probably based on the separate enzymatic mechanism (i.e., RNA polymerase III) that is involved in the synthesis of these RNAs. Our results suggest that the bulk of RNA molecules in the range of 140–770 nucleotides is synthesized by RNA polymerase II, because α-amanitin at 1 μg/ml largely inhibits synthesis in this range. We also find that heparin inhibits (probably by blocking chain initiation) the synthesis of molecules <300 nucleotides in length but permits the synthesis (completion) of larger molecules. On this basis we have distinguished between two subclasses of RNA in the 140–740 nucleotide range (cf. Table 2).

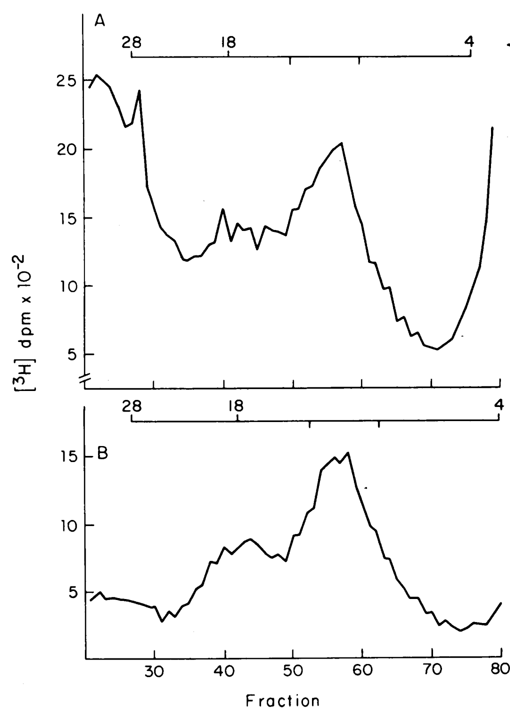

FIG. 4. Definition of a 330–740 nucleotide subclass of DRB-resistant RNA in HeLa cells with the aid of heparin. Cells were prepared and suspended in lysis buffer as described in Fig. 1. Nonidet P40 was added to a concentration of 0.5%, and the suspension was shaken in a Vortex mixer at maximum speed for 1.5 min. The nuclei were collected by centrifugation, washed in 1 ml volumes of lysis buffer, pelleted, drained, and resuspended in Tris/glycerol. Labeling with [^{3}H]UTP was carried out in a volume of 1.0 ml containing nuclei from 4 × 10^7 cells. The 15-min acid-precipitable counts were as follows: control, 12.6 × 10^4 cpm; heparin, 2 mg/ml, 32.7 × 10^4 (260%); DRB, 5.84 × 10^4 (46%); heparin plus DRB, 12.4 × 10^4 (98%). Radioactivity in the heparin plus DRB sample was 38% of that in the heparin sample. RNA was extracted from remaining portions of the samples and analyzed on 2.2% polyacrylamide/1.0% agarose gels (1). (*A*) Heparin. (*B*) Heparin plus DRB.

The major question is whether the DRB resistance of the synthesis of RNA in the 140–740 nucleotide range can be related to the effects of DRB on hnRNA and mRNA synthesis. We will first consider a possible relationship between the DRB-resistant 140–740 nucleotide RNA and the DRB-*sensitive* two-thirds of hnRNA from which the great bulk, if not all, of mRNA is derived. Recent findings concerning adenovirus type 2 transcription in HeLa cells late in infection indicate that 75 μM DRB inhibits the synthesis of virus-specific nuclear RNA by 90–95% and of virus-specific mRNA by >95% (N. Fraser, P. B. Sehgal, and J. E. Darnell, Jr., personal communication). In the nuclei of infected drug-free control cells there is synthesized a large rightward reading virus-specific transcript that comprises close to 85% of the genome. The site of action of DRB appears to be distal to the point of initation of the RNA chain. The synthesis of Sma f (11.6–18.2)-specific RNA molecules, which hybridize to the DNA fragment containing the presumed origin of the long transcript, is resistant to DRB relative to the transcription of fragments downstream. This generates a short virus-specific RNA transcript (about 300–700 nucleotides) that continues to be synthesized in DRB-treated infected cells.

These findings suggest the possibility that, in the synthesis of message-containing transcripts of the cell, too, DRB acts some distance downstream from the point of initiation. Thus, the DRB-resistant RNA chains in the 140–740 nucleotide range may in part represent chains that are transcribed from regions of DNA containing origins for long transcripts that include mRNA sequences. The block in the synthesis of the two-thirds of DRB-sensitve hnRNA could then be explained by action of DRB at a point distal to the point of initation of hnRNA chains. All previous evidence concerning the effect of DRB on initiation of hnRNA chains was obtained under conditions such that the distinction between action at the origin or at a point a few to

Table 2. Three groups of DRB-resistant nucleoplasmic RNA (N = number of nucleotides)

In vitro treatment	Sensitivity to α-amanitin or heparin: 100–120 N	140–330 N	330–740 N
α-Amanitin, 1 μg/ml	Resistant	Sensitive	Sensitive
α-Amanitin, 150 μg/ml	Sensitive	Sensitive	Sensitive
Heparin, 2 mg/ml	Sensitive	Sensitive	Stimulated

Hunan University (222.247.53.42) - 2013/6/13 Download

several hundred nucleotides downstream could not have been made (3, 19–21). If DRB acts by inhibiting RNA polymerase II, the possibility has to be considered that this enzyme may function in two forms, of which the form that initiates a transcript is resistant while a DRB-sensitive form carries out a specific step in transcription at a point downstream.

The question of whether there is a relationship between the DRB-*resistant* one-third of hnRNA and the 140–740 nucleotide RNA is completely open. As yet there is no information concerning the nature of the DRB-resistant one-third of hnRNA except that such RNA is distributed over the entire size range of hnRNA (1, 2), that it is polyadenylylated and capped (2), and that mRNA may not derive from this hnRNA fraction. Herman *et al.* (22) have reported that at least 30% of the poly(A)-adjacent sequences in hnRNA have no apparent counterpart in the cytoplasm. Whether this fraction of poly(A)$^+$ hnRNA molecules, which lacks message sequences, represents the one-third of hnRNA that is resistant to inhibition by DRB remains to be determined. It is of interest that, in the salivary gland cells of *Chironomus tentans,* essentially all of hnRNA synthesis is sensitive to DRB (23, 24). Thus, it appears that the DRB-resistant one-third of hnRNA in HeLa and L cells may be a feature of higher organisms.

There is an extensive literature on more than 10 discrete species of small nuclear RNAs in the size range of 90–300 nucleotides, only a few of which however are NP (25–31). Although the lifetime of the large RNAs is on the order of 10–30 min, some of the small RNA species have half-lives of about 1 day and others show no detectable turnover for several days in exponentially growing tissue culture cells (reviewed in ref. 25). Each species is located in a specific subcellular compartment (27). It has been suggested that these RNAs serve as structural elements in cell architecture in a manner analogous to the structural role of rRNA (27). However, it has also been thought that some small nuclear RNAs may be involved in gene regulatory functions such as gene activation (26). For two NP species, unique and specific linear sequences of nucleotides (26) and modified 5′ termini (32, 33) have been reported. The cap structure in small nuclear RNA is $m_3$2,2,7G-containing cap II, whereas in HeLa cell hnRNA it is m7G-containing cap I (34). Therefore, it is unlikely that small nuclear RNA is related to hnRNA either as a processed byproduct with some regulatory role or as a primer from which to initiate synthesis (34).

There is little information about the number and nature of the NP RNA species in the 330–740 nucleotide range. However, Egyházi *et al.* (35) have described in *Chironomus tentans* a NP RNA species with a molecular weight of 130×10^3 (400 nucleotides), whose synthesis is resistant to DRB. The 400 nucleotide RNA species has been demonstrated in chromosomal fractions as well as in the nuclear sap. The relative amount correlates with the number of bands or DNA content of the different chromosomal fractions.

It is not possible as yet to decide whether cellular RNA species in the 330–770 nucleotide range may play a role in initiation of long transcripts of message-containing RNA. Because RNA molecules in this size range are found in nuclei from cells that had not been treated with DRB, it is clear that not all such molecules become part of hnRNA, even if some may potentially function in forming 5′ segments of primary nuclear transcripts. Clearly, NP DRB-resistant RNA species in the 330–740 nucleotide range, labeled *in vivo,* should be examined for the presence of an m7GpppXmp cap structure and leader sequences (36).

The author thanks Miss Toyoko Kikuchi for her excellent assistance in the conduct of this study, Dr. Pravinkumar B. Sehgal for critical reading of the manuscript, and Drs. Nigel Fraser and James E. Darnell, Jr., for helpful discussions. This investigation was supported by Research Grant CA-18608 and by Program Project Grant CA-18213 awarded by the National Cancer Institute.

1. Tamm, I., Hand, R. & Caliguiri, L. A. (1976) *J. Cell Biol.* **69,** 229–240.
2. Sehgal, P. B., Darnell, J. E., Jr. & Tamm, I. (1976) *Cell* **9,** 473–480.
3. Sehgal, P. B., Derman, E., Molloy, G. R., Tamm, I, & Darnell, J. E. (1976) *Science* **194,** 431–433.
4. Tamm, I. & Sehgal, P. B. (1977), *Adv. Virus Res.,* **22,** 107–258.
5. Eagle, H. (1959) *Science* **130,** 430–437.
6. Penman, S. (1969) in *Fundamental Techniques in Virology,* eds. Habel, K. & Salzman, N. P. (Academic Press, New York), pp. 35–48.
7. Spohr, G. Mirault, M.-E., Imaizumi, T. & Scherrer, K. (1976) *Eur. J. Biochem.* **62,** 313–322.
8. Fedoroff, N., Wellauer, P. K. & Wall, R. (1977) *Cell* **10,** 597–610.
9. Roeder, R. G. (1976) in *RNA Polymerase,* eds. Losick, R. & Chamberlin, M. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 285–329.
10. Price, R. & Penman, S. (1972) *J. Mol. Biol.* **70,** 435–450.
11. Weinmann, R. & Roeder, R. G. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 1790–1794.
12. McReynolds, L. & Penman, S. (1974) *Cell* **1,** 139–145.
13. Udvardy, A. & Seifart, K. H. (1976) *Eur. J. Biochem.* **62,** 353–363.
14. Cox, R. F. (1973) *Eur. J. Biochem.* **39,** 49–61.
15. Ferencz, A. & Seifart, K. H. (1975) *Eur. J. Biochem.* **33,** 605–613.
16. Harmon, S. A. & Gurney, T., Jr. (1976) in *Molecular Mechanisms in the Control of Gene Expression,* eds. Nierlich, D. P., Rutter, W. J. & Fox, C. F. (Academic Press, New York), pp. 361–366.
17. Cook, R. T. & Aikawa, M. (1973) *Exp. Cell Res.* **78,** 257–270.
18. Weinmann, R., Brendler, T. G., Raskas, H. J. & Roeder, R. G. (1976) *Cell* **7,** 557–566.
19. Egyházi, E. (1974), *J. Mol. Biol.* **84,** 173–183.
20. Egyházi, E. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 947–950.
21. Egyházi, E. (1976) *Nature* **262,** 319–321.
22. Herman, R. C., Williams, J. G. & Penman, S. (1976) *Cell* **7,** 429–437.
23. Egyházi, E., Daneholt, B., Edström, J.-E., Lambert, B. & Ringborg, U. (1970) *J. Cell Biol.* **47,** 516–520.
24. Ringborg, U. & Rydlander, L. (1971) *J. Cell Biol.* **51,** 355–368.
25. Weinberg, R. A. (1973) *Annu. Rev. Biochem.* **42,** 329–354.
26. Ro-Choi, T. S. & Busch, H. (1974) in *The Cell Nucleus,* ed. Busch, H. (Academic Press, New York), Vol. III, pp. 151–208.
27. Zieve, G. & Penman, S. (1976) *Cell* **8,** 19–31.
28. Marzluff, W. F., Jr., White, E. L., Benjamin, R. & Huang, R. C. (1975) *Biochemistry* **14,** 3715–3724.
29. Hellung-Larsen, P. & Frederiksen, S. (1975) *Int. J. Biochem.* **6,** 361–370.
30. Frederiksen, S. & Hellung-Larsen, P. (1975) *FEBS Lett.* **58,** 374–378.
31. Sklar, V. E. F. & Roeder, R. G. (1977) *Cell* **10,** 405–414.
32. Ro-Choi, T. S., Choi, Y. C., Henning, D., McCloskey, J. & Busch, H. (1975) *J. Biol. Chem.* **250,** 3921–3928.
33. Cory, S. & Adams, J. M. (1975) *Mol. Biol. Rep.* **2,** 287–294.
34. Fernandez-Munoz, R., Lavi, U. & Darnell, J. E. (1977) *Nucleic Acid Res.,* in press.
35. Egyházi, E., Daneholt, B., Edström, J.-E., Lambert, B. & Ringborg, U. (1971) *J. Cell Biol.* **48,** 120–127.
36. Klessig, D. F. (1977) *Cell,* **12,** 9–21.

Hunan University (222.247.53.42) - 2013/6/13 Download