Short Capped hnRNA Precursor Chains in HeLa Cells: Continued Synthesis in the Presence of 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole†

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ABSTRACT: The labeling of $m^7GpppN_1mpN_2p$ caps with L-[*methyl*-^{3}H]methionine on short (100–500 nucleotides) heterogeneous nuclear RNA (hnRNA) chains of HeLa cells is increased 2–3 times but the labeling of caps on longer (>2000 nucleotides) hnRNA chains is decreased by ~80% by treatment of the HeLa cells with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). The experimental conditions were as follows: HeLa cells were treated with 75 μM DRB for 40 min before labeling and also during the 30-min pulse of L-[*methyl*-^{3}H]methionine; actinomycin D (0.05 μg/mL) was used to suppress ribosomal RNA synthesis. Control cells received no DRB. The RNA was separated in Me_2SO gradients to ensure no aggregation. Labeling of cells with [^{3}H]uridine for 10 min and separation of RNA by these techniques reconfirmed the findings [Tamm, I., Hand, R., & Caliguiri, L. A. (1976) *J. Cell Biol. 69*, 229–240; Sehgal, P. B., Darnell, J. E., Jr., & Tamm, I. (1976) *Cell 9*, 473–480] that 70–80% of the synthesis of hnRNA (>1000 nucleotides) is sensitive to inhibition by DRB but that 20–30% is resistant. This analysis of the methyl-labeled caps provides evidence that DRB causes early termination of a large fraction (~70–80%) of hnRNA precursor chains. In contrast to the finding of continued synthesis and accumulation of short $m^7GpppN_1mpN_2p$-capped chains in the presence of DRB, the synthesis of $m^{2,2,7}GpppN_1mpN_2mp$-capped small nuclear RNAs was inhibited by ~70% by DRB.

5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) accentuates early termination of cellular (HeLa) RNA chains made by RNA polymerase II (Tamm, 1977; Tamm & Kikuchi, 1979). The available evidence suggests that the inhibition of the synthesis of two-thirds of heterogeneous nuclear RNA (hnRNA) and >95% of mRNA (Tamm et al., 1976; Sehgal et al., 1976) by DRB is due to early termination of the bulk of hnRNA transcripts representing presumptive mRNA precursor chains. That DRB accentuates premature termination of both "early" and "late" transcripts from the adenovirus genome has been fully documented (Fraser et al., 1978, 1979; Sehgal et al., 1979). Thus far there has been no evidence that DRB interferes with capping, polyadenylation, or processing of RNA transcripts (Sehgal et al., 1976; Fraser et al., 1979; reviewed in Tamm & Sehgal, 1979b).

Since the addition of 5′ cap structures occurs early in hnRNA synthesis, as has recently been shown in HeLa (Sommer et al., 1978) and Chinese hamster ovary (CHO) cells (Salditt-Georgieff et al., 1980), the short RNA chains, which are made in undiminished amounts and which accumulate in the presence of DRB, would be expected to contain cap I ($m^7GpppN_1mpN_2p$) oligonucleotide structures characteristic of hnRNA. Thus, measurement of short capped hnRNA precursors represents an approach to monitoring premature termination of hnRNA transcripts.

We report in the present communication that short chains capped with 5′ cap I structures containing m^7G and all 4 bases in the N_1 position are 2–3 times more abundant in DRB-treated cells than in control HeLa cells. In contrast, cap II structures characteristic of certain of the homodisperse small nuclear RNAs [such as U_1 and U_2 in the terminology of Ro-Choi & Busch (1974) or D and C in the terminology of Weinberg & Penman (1968)] are decreased by ~75% in DRB-treated cells.

Materials and Methods

Cell Culture. HeLa S3 (human carcinoma derived) cells were grown in suspension culture in the spinner modification of Eagle's minimal essential medium (Eagle, 1959) supplemented with 5% fetal calf serum (FCS). They were diluted 1:2 or 1:4 to maintain the density between 2×10^5 and 4×10^5 cells/mL.

Treatment, Labeling, and Fractionation of Cells. We used 10^8 cells per sample in 50 mL of spinner medium containing 5% FCS. Control and DRB (75 μM) treated cells were incubated for 40 min at 37 °C before labeling. For 25 min before labeling both cell samples were treated with actinomycin D (0.05 μg/mL). The cells were then centrifuged, washed once with methionine-free medium, resuspended at a concentration of 2×10^6 cells/mL in methionine-free medium (still containing inhibitors as noted), and supplemented with adenine (0.02 mM), guanosine (0.02 mM), and sodium formate (0.01 mM) to suppress nonmethyl purine ring labeling (Maden et al., 1972; Desrosiers et al., 1974). The methionine-free medium was Eagle's minimal essential medium modified by Joklik (Grand Island Biological Co.). It was supplemented with 5% dialyzed FCS. Cells were labeled for 30 min with L-[*methyl*-^{3}H]methionine (specific activity 11.0 Ci/mmol; New England Nuclear Corporation), 150 μCi/mL, in the continued presence or absence of DRB (75 μM). Actinomycin D (0.05 μg/mL) was present during labeling. Cells were collected by centrifugation, washed once in phosphate-buffered saline (PBS), and lysed in 0.5% NP-40 in 2 mL of isotonic, high pH buffer (0.14 M NaCl, 0.0015 M $MgCl_2$, and 0.01 M Tris, pH 8.4) on ice (5 min) with intermittent vortex treatment. The nuclei were washed once with the same buffer and lysed in 2 mL of high salt buffer (0.5 M NaCl, 0.05 M $MgCl_2$, and 0.01 M Tris, pH 7.4) containing proteinase-free and ribonuclease-free (PFRF) DNase (Wang & Moore, 1978) (40 μg/mL). $NaDodSO_4$, EDTA, and sodium acetate were added to final concentrations of 0.5%, 50 mM, and 0.05 M, respectively. The nuclear lysate was then diluted to 7 mL with 0.05 M sodium acetate buffer, pH 5.1, containing 10 mM EDTA and 0.2% $NaDodSO_4$.

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RNA Extraction and Analysis of Caps. The RNA was extracted twice with phenol and chloroform at 65 °C (Soiero & Darnell, 1969), washed twice with chloroform, and precipitated with ethanol at −20 °C. Linear (5–20% w/v) sucrose gradients were prepared in 99% Me_2SO containing 0.01 M Tris, pH 7.4, and 0.001 M EDTA (TE) (Derman et al., 1976). RNA samples were resuspended in 0.1 mL of TE, and 0.4 mL of Me_2SO/TE was added. They were heated at 65 °C for 2 min, layered on gradients, and centrifuged in an SW27 rotor at 24 000 rpm for 74 h at 28 °C. After centrifugation, 1-mL fractions were collected. A portion (0.1 mL) of each fraction was precipitated with 10% trichloroacetic acid after addition of bovine serum albumin (0.1 μg/mL) as carrier and counted in a scintillation spectrometer. Appropriate fractions were then pooled (see legend for Figure 1) and yeast tRNA was added as carrier (2 μg/mL). The pooled fractions were diluted with equal volumes of NETS buffer (0.2 M NaCl, 0.01 M EDTA, 0.01 M Tris, pH 7.4, and 0.2% $NaDodSO_4$) and precipitated with 3 volumes of ethanol. Samples were digested with pancreatic RNase A (Worthington Biochemical Corp.) (lower portion of each gradient, 10 μg; upper portion, 150 μg) and RNase T2 (Calbiochem) (lower portion, 10 units; upper portion, 60 units) in 0.015 M ammonium acetate buffer, pH 4.6, and 2 mM EDTA for 8–15 h at 37 °C. The digests were diluted with 0.02 M Tris, pH 7.4, and applied to DEAE-52 columns equilibrated with 0.02 M Tris, pH 7.4. Yeast tRNA digested with pancreatic RNase A was used as a marker, which gave positions of molecules with different charge, detected by absorbance at 254 nm. Elution was carried out with a gradient created by mixing 100 mL each of 0.02 M Tris–7 M urea, pH 7.4, and 0.3 M NaCl–0.02 M Tris–7 M urea, pH 7.4. Fractions (1 mL) were collected, and one-half of each fraction was counted using formula 950A scintillant (New England Nuclear). Appropriate fractions were pooled (see Figure 2), diluted with 2 volumes of 0.01 M ammonium bicarbonate buffer (pH 7.5), applied to DEAE-52 columns equilibrated with the ammonium bicarbonate buffer, and eluted with 20% triethylammonium bicarbonate (maximal pH 8.6). The eluates were lyophilized, and the preparations were washed twice with distilled water and then sequentially digested with Penicillium nuclease (P1) (Furuichi et al., 1975; Yamasa Ltd.) (500 μg/mL) in 0.01 M sodium acetate buffer (pH 6.0) containing 5 mM $MgCl_2$, with nucleotide pyrophosphatase (Sigma Chemical Co.) (1.0–1.5 μg/mL), and with bacterial alkaline phosphatase (BAP) (Worthington Biochemical Corp.) (20 μg/mL) in 0.02 M Tris (pH 7.6). Each digestion was carried out at 37 °C for 30 min. Ethylene glycol was added to the digests to a concentration of 20%, and the digests were analyzed by high-pressure liquid chromatography (high-pressure LC) at 42 °C (Pike & Rottman, 1974; Desrosiers et al., 1974; Rottman et al., 1976) on an Aminex A9 column (Salditt-Georgieff et al., 1980). The liquid phase was 0.4 M ammonium formate (pH 4.25) with 20% ethylene glycol, the pressure was 2500–2800 psi, and the flow rate was 0.5 mL/min. Fractions of 0.25 mL were collected through fraction number 70; the remaining 50 were 0.5-mL fractions. The fractions were counted with formula 950A scintillant in a scintillation spectrometer.

Results

Size Distribution of RNA Labeled with L-[*methyl*-^{3}H]-*Methionine in Control and DRB-Treated Cells.* The sucrose–Me_2SO gradient profiles in Figure 1A,B show the effect that treatment of HeLa cells with 75 μM DRB has on the labeling of nuclear RNA with L-[*methyl*-^{3}H]methionine in the presence of actinomycin (0.05 μg/mL). While the labeling

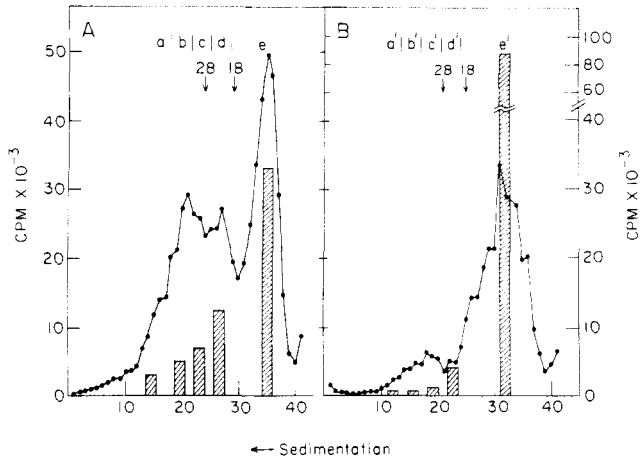

FIGURE 1: Sucrose–Me_2SO gradient profiles of nuclear RNA labeled with L-[*methyl*-^{3}H]methionine (30 min) in control and DRB-treated cells and the abundance of cap I structures in RNA of various sizes. For experimental procedures see Materials and Methods. (A) Control; (B) DRB (75 μM, present for 40 min before labeling and also during labeling). For analysis of cap structures, gradients were divided as indicated: a, b, c, and d refer to portions of the gradient in the >18S region; e refers to the <18S portion. The bar graphs represent m^7G-containing caps as determined in Figure 2. The cpm × 10^{-3} values on the left and right ordinates refer both to the cpm in sucrose–Me_2SO gradient fractions and to the total cpm in m^7G-containing caps in pools of fractions indicated by the graphs.

of <18S RNA was only slightly reduced, that of >18S RNA was markedly decreased in DRB-treated cells. The majority of methyl-labeled <18S RNA (Figure 1) appears to be from 100 to 500 nucleotides in length in both control and DRB-treated cells. Figure 1, A and B, is also used to show, in summary form, the abundance of cap I structures in ≤18S RNA and in four size categories of >18S RNA. The bar graphs are based on results of cap analysis described below. It is evident that cap I structures in the ≤18S RNA are approximately 3 times more abundant in the DRB-treated as compared to the control cells. In sharp contrast, labeling of cap I structures in >18S RNA in the DRB-treated cells is reduced, on the average, by 80%.

Cap Structures in Short RNA Chains. The primary question addressed in these experiments concerns the nature of the cap structures in the short RNA chains. Two kinds of labeled caps exist in nuclear RNA labeled to steady state: (a) cap I structures of the type $m^7GpppN_1mpN_2p$ (−4.5 to −5.5 charge), forming 5′ termini in chains related to hnRNA (Perry et al., 1975; Salditt-Georgieff et al., 1976, 1980); (b) cap II structures of the type $m^{2,2,7}GpppAmpUmpNp$ (−5.5 to −6.5 charge), forming 5′ termini in certain homodisperse small nuclear RNA species (Ro-Choi & Busch, 1974; Fernandez-Munoz et al., 1977). In addition, some cap I structures containing $m^{2,2,7}G$ have been detected in the short, briefly labeled nuclear RNA chains of CHO cells, presumably precursors to the finished cap II containing $m^{2,2,7}G$ (Salditt-Georgieff et al., 1980).

Figure 2A shows that DEAE-52 column chromatography of the enzymatic digest of the ≤18S control RNA from the sucrose–Me_2SO gradient resolves two adjacent peaks representing −4.5- to −5.5- and −5.5- to −6.5-charge cap structures, respectively. In the preparation from DRB-treated cells, there is a large peak corresponding to −4.5- to −5.5-charge cap I structures but only a shoulder corresponding to −5.5- to −6.5-charge cap II structures.

Figure 2A′,A″ shows the results of high-pressure LC analysis of the cap I and cap II structures, respectively, from control cells. Both m^7G and $m^{2,2,7}G$ were present in the −4.5-

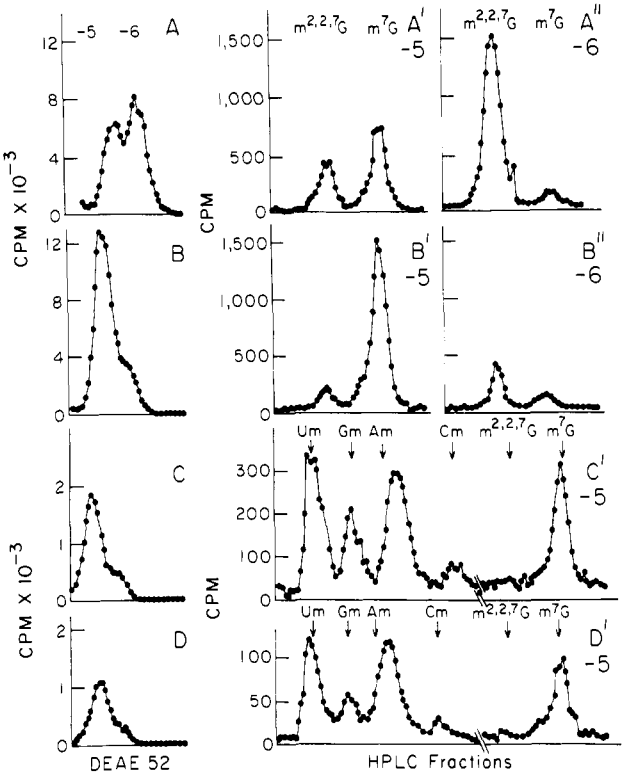

FIGURE 2: Methyl-labeled cap structures in pancreatic RNase A and RNase T2 digests of hnRNA chains analyzed by DEAE column chromatography (left, panels A–D); further analyses by high-pressure liquid chromatography of the nucleoside composition after P1, nucleotide pyrophosphatase, and BAP digestion (right, panels A′–D′, A″ and B″). Digests of hnRNA chains <2000 nucleotides in length: (A (control) and B (DRB)) nucleotides in charge regions −5 and −6 (only the cap I and II peaks of the DEAE-52 chromatograms are shown); (A′ and B′) nucleosides from charge region −5; (A″ and B″) nucleosides from charge region −6 (only the $m^{2,2,7}G$ and m^7G peaks of the high-pressure LC chromatograms are shown). Digests of hnRNA chains 2000–4000 nucleotides in length: (C (control) and D (DRB)) nucleotides in charge regions −5 and −6; (C′ and D′) nucleosides from charge region −5. For experimental procedures see Materials and Methods. The molar fractions of m^7G and $m^{2,2,7}G$ caps in short (<2000 nucleotides long) RNA chains, based on high-pressure LC, are given in Table I. In RNA 2000–4000 nucleotides long, virtually no cap II structures were detected by DEAE chromatography (panels C and D), and no caps containing $m^{2,2,7}G$ were detected by high-pressure LC. The even larger molecules (>4000 nucleotides long; cf. fractions a, b, and c in Figure 1) also contained no cap II structures and are therefore assumed to contain no $m^{2,2,7}G$.

to −5.5-charge material, whereas $m^{2,2,7}G$ was the predominant nucleoside in the −5.5- to −6.5-charge material. Thus, under the conditions of 30-min labeling, there was extensive incorporation of L-[*methyl*-^{3}H]methionine not only into the m^7G-containing cap I structures but also into the $m^{2,2,7}G$-containing cap II structures in control cells. The −4.5- to −5.5-peak region in the DEAE-52 chromatographic analysis comprises predominantly m^7G-containing cap I structures but probably also some $m^{2,2,7}G$-containing cap I structures and possibly a limited admixture of $m^{2,2,7}G$-containing cap II structures.

High-pressure LC permits accurate estimation of the relative amounts of m^7G-containing vs. $m^{2,2,7}G$-containing cap structures in the short RNA chains; calculations which take into account the different levels of labeling of these structures indicate an $m^7G/m^{2,2,7}G$ ratio of 1.3 in control cells (Table I).

High-pressure LC of the −4.5- to −5.5-charge and the −5.5- to −6.5-charge materials from DRB-treated cells shows (see Figure 2B′,B″) that m^7G-containing cap structures greatly predominate in short chains after DRB treatment, as indicated

Table I: Molar Fraction of m^7G and $m^{2,2,7}G$ Caps from Short Nuclear RNA[a]

	m^7G		$m^{2,2,7}G$	
	cpm	molar fraction	cpm	molar fraction
control	6360	0.57	14400	0.43
DRB	11900	0.89	4240	0.11

[a] Cap I and cap II structures were obtained by DEAE chromatography from digests of nuclear RNA <2000 nucleotides long (see Figure 1 and Figure 2) and analyzed by high-pressure LC (see Figure 2). The molar fractions were calculated assuming three times as many cpm in $m^{2,2,7}G$ per mole as in m^7G.

Table II: DEAE-52 Column Chromatography of hnRNA of Various Sizes

size category[a]	control (cpm)			DRB (cpm)			DRB (% of control)		
	−2[b]	−3	−5	−2	−3	−5	−2	−3	−5
a	32400	29600	2970	3970	7150	618	12	24	21
b	23300	4000	5270	1330	6790	588	6	13	11
c	19800	39900	6760	1690	11300	1130	9	28	17
d	45800	31700	12110	5960	5670	4230	13	18	35
mean							10	21	21

[a] See Figure 1. [b] Negative numbers refer to charge regions.

by an $m^7G/m^{2,2,7}G$ ratio of 8.3. Thus, as shown in Table I, DRB caused an approximately 2-fold increase in m^7G-capped short chains but at the same time also a 3-fold decrease in the $m^{2,2,7}G$-capped short chains.

Cap Structures in RNA Chains of Intermediate Lengths and Longer. As indicated in Figure 1A,B >18S chains were divided into four size categories (a, b, c, and d) and analyzed for the presence of cap structures. DEAE-52 column chromatography showed (see Figure 2C,D) that in both control and DRB-treated cells the −4.5- to −5.5-charge cap I structures were predominant in the size d category chains (2000–4000 nucleotides in length). This confirms the effectiveness of the Me_2SO gradient for separation of small chains from larger chains. High-pressure LC revealed the presence of considerable amounts of m^7G but very little $m^{2,2,7}G$ in larger molecules. DRB decreased labeling of m^7G by about two-thirds in these RNA chains of intermediate length (category d). The reduction in the labeling of the other nucleosides in cap structures was of a similar magnitude. It should be noted that there was no peak of labeled Am detected in the high-pressure LC analysis of cap I structures but rather a peak of material which eluted slightly after the Am OD marker in the position expected for m^6Am. This compound is a known constituent of mRNA and hnRNA caps (Perry et al., 1975; Rottman et al., 1976; Salditt-Georgieff et al., 1976, 1980). Because a large number of caps have m^6Am in the N_1 position, the percentage of total cap counts in m^7G after methyl labeling would be expected to be <50%. In CHO cell hnRNA, 32–34% of the total cap counts are in m^7G (Salditt-Georgieff et al., 1980). In the present study we have not critically determined this percentage or investigated the relatively high counts in the region of Um but have used high-pressure LC purely to determine the $m^7G/m^{2,2,7}G$ ratio.

In view of the virtual absence of $m^{2,2,7}G$ in cap I structures obtained from the intermediate-sized RNA chains (category d in Figure 1), the total methyl-derived radioactivity in the nucleotides with a −4.5 to −5.5 charge can be used to determine the effect of DRB on the labeling of hnRNA type cap I structures in long (>28 S) RNA chains as well as in chains of intermediate length (~18–28 S). Table II shows that DRB

decreases labeling of cap I structures in >18S hnRNA chains by ~80%. This provides new evidence that the same proportion of the DRB-resistant >18S hnRNA is capped at the 5′ end as is total cell RNA labeled by [^{3}H]uridine. A similar conclusion was suggested previously (Sehgal et al., 1976; Sehgal, 1977; quoted in Tamm & Sehgal, 1978). Most of the −3-charge material is probably derived from ribosomal RNA and reflects 2′-O-methylation of molecules that escaped inhibition by actinomycin D (Salditt-Georgieff et al., 1976; Schibler & Perry, 1976). The −2-charge material is to an unknown extent accounted for by m^{6}Ap in hnRNA (Salditt-Georgieff et al., 1976). Nevertheless, there is ~90% suppression of the −2-charge material, suggesting the possibility that labeling of m^{6}Ap in hnRNA with [*methyl*-^{3}H]methionine is more inhibited by DRB than labeling of hnRNA with [^{3}H]uridine.

The bar graph in Figure 1, which summarizes the distribution of cap I structures in hnRNA of various sizes, was constructed as follows: (1) for gradient parts a, b, c, and d, the sum of label in the nucleotides with a charge of −4.5 to −5.5 was assumed to represent m^{7}GpppN$_1$m-related counts because no m^{2,2,7}G was recovered from even the shortest chains (d) among these samples; (2) for gradient part e, the m^{7}GpppN$_1$m counts were calculated after high-pressure LC separation of the methylated nucleosides derived from the −4.5- to −5.5-charged structures on the assumption that the bulk of m^{2,2,7}G-associated radioactivity in the −5-charge material was derived from cap I structures whose N_2 has not yet been methylated.

$$\text{cpm in m}^7\text{G} + 1 \times \text{cpm in m}^7\text{G} = \text{cpm in m}^7\text{GpppN}_1\text{m}$$

$$\text{cpm in m}^{2,2,7}\text{G} + 1/3 \times \text{cpm in m}^{2,2,7}\text{G} = \text{cpm in m}^{2,2,7}\text{GpppN}_1\text{m}$$

$$\frac{\text{cpm in m}^7\text{GpppN}_1\text{m}}{\text{cpm in m}^7\text{GpppN}_1\text{m} + \text{cpm in m}^{2,2,7}\text{GpppN}_1\text{m}} = \text{fraction of the total high-pressure LC cap I cpm in m}^7\text{GpppN}_1\text{m (for control = 0.73; for DRB = 0.91)}$$

For control: DEAE-52 −5 peak 49916 cpm × 0.73 = 36439, total cpm in m^{7}GpppN$_1$m. For DRB: DEAE-52 −5 peak 96358 cmp × 0.91 = 87686, total cpm in m^{7}GpppN$_1$m.

Size Profiles of [^{3}H]Uridine-Labeled RNA from Control and DRB-Treated Cells. For comparison with the L-[*methyl*-^{3}H]methionine-labeled profiles, we labeled HeLa cells with [^{3}H]uridine for 10 min after treatment of the cells as described under Materials and Methods. Nuclear RNA was isolated and analyzed on sucrose–Me_2SO gradients. Figure 3 shows that labeling of nuclear RNA with [^{3}H]uridine under conditions which approximate steady-state labeling conditions gives a profile which is dominated by the extensive incorporation of label into long (>28 S) hnRNA chains (Derman et al., 1976). Peaks of short RNA in the 100–500-nucleotide range (cf. Figure 1) are not discernible, presumably because of the rapid and continuous incorporation of [^{3}H]uridine into chains growing from short to long. The active chain elongation obscures the fraction of prematurely terminated chains in control cells as well as in DRB-treated cells in spite of the fact that in the latter the extent of premature termination is such that synthesis of hnRNA is restricted to 20–30% of that in control cells. As reported previously (Tamm et al., 1976; Sehgal et al., 1976; Tamm & Sehgal, 1979b), the DRB-resistant, >1000 nucleotides long, hnRNA is distributed over the entire size range of hnRNA. Using [^{3}H]uridine under nascent chain labeling conditions in vivo (pulse times <1 min),

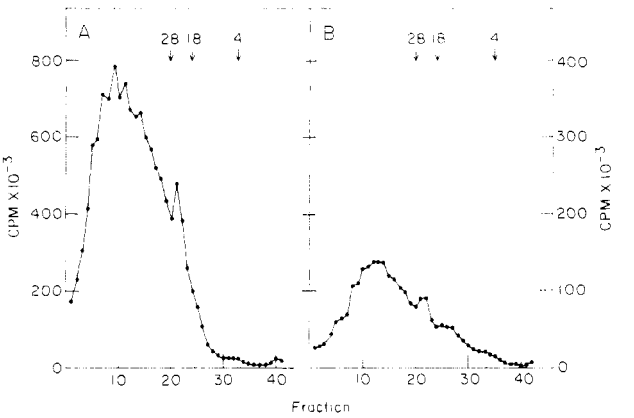

FIGURE 3: Sucrose–Me_2SO gradient profiles of hnRNA labeled with [^{3}H]uridine (10 min) in control and DRB-treated cells. For general procedure see Materials and Methods. The medium contained L-methionine, 2 mg/L. [^{3}H]Uridine (specific activity 27.6 Ci/mmol; New England Nuclear Corporation) was used at a concentration of 10 μCi/mL. (A) Control; (B) DRB (75 μM, present for 40 min before labeling and also during labeling). Incorporation of [^{3}H]uridine into RNA in DRB-treated cells was corrected by a factor of 3.45 for reduced transport of the precursor (Tamm et al., 1976; Sehgal & Tamm, 1976). The factor used was calculated from total cell-associated radioactivity, which was 123 × 10^6 cpm in the control sample and 35 × 10^6 cpm in DRB (29%). Based on corrected acid-precipitable counts in isolated RNA (control 12.5 × 10^6 and DRB 0.50 × 10^6), DRB caused 80% inhibition of RNA synthesis.

it has been possible to detect the population of abundant, short, and apparently prematurely terminated chains in DRB-treated HeLa cells (Tamm & Kikuchi, unpublished results). In CHO cells not treated with DRB, [^{3}H]uridine labeling of nascent chains for 10 or 20 s also revealed a population of short, apparently prematurely terminated chains (Salditt-Georgieff et al., 1980).

Discussion

We have demonstrated that in the nuclei of HeLa cells treated with DRB there accumulate short chains of RNA capped at their 5′ ends with m^{7}GpppN$_1$m structures. These findings provide strong support for the view that DRB accentuates premature termination of hnRNA precursor chains (Tamm, 1977; Tamm & Kikuchi, 1979; Tamm & Sehgal, 1979a,b). It thus appears that DRB acts on the synthesis of cellular message-containing transcripts in the same manner as it does on adenovirus transcription (Fraser et al., 1978, 1979; Sehgal et al., 1979).

Present results and those obtained previously in CHO cells not treated with DRB (Salditt-Georgieff et al., 1980) provide a basis for quantitative determination of the premature termination of hnRNA precursor chains. This opens up an approach to the investigation of premature termination as a possible mechanism whereby mammalian cells may regulate transcription in a variety of different circumstances (e.g., during development, in response to hormone action or variable nutritional conditions, or as part of the cellular aging process). As yet there appears to be no evidence that attenuation of transcription through premature termination is a mechanism of transcriptional regulation in mammalian cells. Presumably, the premature termination that depends on coupled transcription–translation in the adaptation of *E. coli* cells to variable amino acid levels (Zurawski et al., 1978; DiNocera et al., 1978; Barnes, 1978) cannot occur in animal cell nuclei. This close physical linkage of transcription to translation in prokaryotes has no counterpart in mammalian cells. However, the premature termination that occurs in transcription of the λ bacteriophage genome depends only on the function of a λ

gene product (Ptashne, 1978; Rosenberg et al., 1978). This model could conceivably have a counterpart in eukaryotic transcription. Therefore, the possible functional significance of such termination should be investigated.

In the adenovirus system (Fraser et al., 1978, 1979; Sehgal et al., 1979) and also in the transcription of the cellular genome, DRB can be used to accumulate short chains of promoter-proximal RNA. The investigation of promoters and sequence homologies near the 5′ termini of different transcription units is thereby facilitated. The fact that short capped chains accumulate in DRB-treated cells in quantities which exceed those in untreated cells indicates that the turnover of such chains is not significantly more rapid than the combined turnover and processing of the larger hnRNA chains. Indeed it has been demonstrated for specific adenovirus transcription units that RNA chains of discrete sizes, representing prematurely terminated promoter-proximal transcripts, accumulate in DRB-treated cells and form prominent bands in high-resolution electropherograms (Fraser et al., 1979). Many such bands have much less prominent counterparts in adenovirus-infected cells not treated with DRB.

In the present work we obtained evidence that the synthesis of short RNA capped with cap II structures of the type $m^{2,2,7}GpppN_1mN_2m$ is inhibited by ~70% by 75 μM DRB. This finding is in complete agreement with recent evidence that DRB inhibits the synthesis of U1 and U2 (D and C) small homodisperse nuclear RNA species (Hellung-Larsen, 1979, personal communication). It has been reported that the U1 and U2 (D and C) species of small nuclear RNA are transcribed by RNA polymerase II (Frederiksen et al., 1978; Frederiksen & Hellung-Larsen, 1979) which also transcribes hnRNA (Roeder, 1976). Results of UV inactivation studies indicate that C and D are derived from transcription units of large size which may be up to 5000 nucleotides long (Eliceiri, 1979). They occur in association with ribonucleoprotein particles containing hnRNA (Sekeris & Niessing, 1975; Deimel et al., 1977). Small, metabolically stable nuclear RNAs, tentatively identified as species C, D, and G, have recently been reported to be covalently linked to chromosomal DNA in HeLa cells (Pederson & Bhorjee, 1979). It is noteworthy that the extent of inhibition of synthesis of $m^{2,2,7}G$-capped short chains by DRB is about equal to that of inhibition of hnRNA that is >1000 nucleotides long (Tamm et al., 1976; Sehgal et al., 1976; and present results from labeling experiments with [^{3}H]uridine).

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Pertinence of Nuclear Envelope Nucleoside Triphosphatase Activity to Ribonucleic Acid Transport†

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ABSTRACT: Nuclear envelopes were isolated from purified rat and calf liver nuclei via different methods. Basic characterization of nuclear envelopes demonstrated phospholipid distributions similar to those found in microsomes, but enzymatic compositions and fatty acid moieties of phosphatidylserine differed from those in microsomes. A substantial Mg^{2+}-dependent NTPase was found in nuclear envelopes from both sources. The activity was linear with protein concentration and showed a sharp pH dependency with maximal activity near pH 7.5. Arrhenius analysis of the activity in rat liver preparations disclosed an activation energy of 13.8 kcal/mol, and Lineweaver–Burk plots showed a K_m of 1.8 mM ATP. Under similar conditions, calf liver preparations showed an activation energy of 13.3 kcal/mol and a K_m of 1.9 mM ATP; with Mg^{2+} added in 5 mM excess (over nucleotide concentration) they yielded linear Eadie plots. The NTPase activity in nuclear envelopes from both sources showed a broad substrate specificity and induced declines in the energy charge of various nucleotide additives that paralleled stimulation of RNA transport in vitro by these additives. Nuclear envelopes from both sources were able to hydrolyze the high-energy phosphate bonds of diphosphate nucleotides. The ability to utilize ADP was not dependent on coupled oxidative phosphorylation or on electron transport; rather, it apparently proceeds via a myokinase-like activity that furnishes ATP. A number of agents that modify RNA transport in vitro similarly modified the NTPase activity. For instance, cAMP increases RNA transport and the NTPase activity, and further investigation showed that cAMP increased the K_m of the NTPase activity only slightly and the V_{max} by 65%. Further studies in vivo following CCl_4 or thioacetamide treatment of rats demonstrated a parallelism between alterations in RNA transport in vivo and nuclear envelope NTPase activity. Histochemical studies demonstrated that the NTPase activity was distributed along the nuclear envelope and was not localized to nuclear pores under the conditions employed. Supporting this result, we found that thioacetamide-induced nuclear swelling produces changes in nuclear envelope surface area which parallel increases in the nuclear envelope NTPase activity produced by this treatment. The reciprocity between the NTPase activity in nuclear envelopes and RNA transport, with regard to substrate behavior and to effects of activators and inhibitors and perturbations induced by in vivo treatments, suggests that this activity participates in RNA transport.

Isolated nuclei have been used in vitro to study nucleocytoplasmic RNA transport to a surrogate cytoplasm. A potential problem with such systems is to what extent the "selection" of RNA transcripts for transport from isolated nuclei in vitro reflects the corresponding process in vivo. There are, however, many aspects of this model that show a degree of biological reproducibility. Many characteristics of the RNA transported from isolated nuclei, including poly(A)[1] content (Schumm & Webb, 1974; Smuckler & Koplitz, 1976), size (Smuckler & Koplitz, 1976; Ishikawa et al., 1970a), base content and activity in directing protein synthesis in vitro (Ishikawa et al., 1970a), incorporation into polysomes in reconstructed systems (Ishikawa et al., 1970b), and inclusion in specific RNP particles (Smuckler & Koplitz, 1974; Raskas, 1971; Ishikawa et al., 1969; Sato et al., 1977), point to similarities between RNA transported in vitro and messenger and ribosomal RNA found in the cytoplasm. RNA transport proceeds in vitro with an activation energy of 13 kcal/mol (Clawson & Smuckler, 1978), a value consistent with an energy-requiring process. The constancy of this value under incubation conditions which dramatically alter the species of RNA transported suggests that the system's selectivity is distinct from its energetics. Furthermore, the relationship between facilitated RNA transport and the hydrolysis of high-energy phosphate bonds is of high statistical significance (Clawson et al., 1978). One high-energy phosphate bond is hydrolyzed in the facilitated transport of each nucleotide in transported RNA.

RNA transport in vitro necessarily involves both intranuclear RNA processing and subsequent transport. Other investigators have reported that RNA is processed in the absence of an energy source (Brunner & Raskas, 1972), and the results of our work support this observation. When isolated nuclei were incubated in surrogate cytoplasmic mixtures containing AMPCP (a methylene-blocked analogue of ADP which does not possess a high-energy phosphate bond and does not stimulate RNA transport), nuclear RNA (rapidly labeled with [^{14}C]orotic acid or L-[^{3}H]methionine) was processed but not transported (unpublished data). These findings suggest that it is the translocation of RNA from the nuclear interior which

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[1] Abbreviations used: NE, nuclear envelope; RNP, ribonucleoprotein; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; $NaDodSO_4$, sodium dodecyl sulfate; TKM buffer, 50 mM Tris-HCl (pH 7.4), 25 mM KCl, and 5 mM $MgCl_2$; NTPase, nucleoside triphosphatase; DNP, 2,4-dinitrophenol; BHT, butylated hydroxytoluene; poly(A), poly(adenylic acid).

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