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Synthesis of actinomycin-insensitive RNA during the first post-irradiation mitotic cycle, in the synchronously mitotic plasmodia of *Physarum polycephalum*

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Abstract. A sucrose density gradient analysis of ^{3}H-uridine pulse-labelled RNA from the first postirradiation mitotic cycle of *Physarum polycephalum* shows that all the density classes of RNA synthesized during this period are resistant to the peptide-antibiotic, actinomycin D. In fact, the synthesis is found to be greater in the presence of the drug. The heterogenously sedimenting synthetic activity here may represent a single species of RNA and its precursors or more than one kind of RNA. Further characterization of this RNA is meaningful in view of the actinomycin insensitivity of the postirradiation mitotic cycle itself to this antibiotic.

Keywords. Ultraviolet-irradiation; transcription; actinomycin-resistance; postirradiation mitotic cycle; *Physarum polycephalum.*

1. Introduction

Nuclear divisions in the multinucleated, syncytial plasmodia of the myxomycete, *Physarum polycephalum,* are synchronous and rhythmic in nature. The multinucleated state makes the plasmodia highly resistant to radiation. Because of the above characteristics, this organism is ideal for studying cycle-dependent radiation effects other than mere cell killing. It is known that ultraviolet (UV)-irradiation of the plasmodia induces long mitotic delays (Sachsenmaier 1966; Devi *et al* 1968a; Sachsenmaier *et al* 1970; Kumari and Nair 1984) and also causes the degeneration of a large number of nuclei (Devi *et al* 1968a; Devi and Guttes 1972). The degeneration of the nuclei in turn leads to a significant alteration of the nucleo-cytoplasmic ratio in this syncytial system, and we had measured this change in terms of total cellular RNA/DNA and protein/DNA ratios (Devi *et al* 1968a; Devi and Guttes 1972). Apparently, to adjust this altered nucleo-cytoplasmic ratio, the delayed first postirradiation mitosis (PIM) is followed by a few shorter-than-normal mitotic cycles, during which time the above ratios return to the control level. It was also observed that the delayed first PIM and the cycle after that are resistant to continuous treatment with actinomycin D (200 μg ml^{-1}), an antitumor, peptide-antibiotic (Devi and Guttes 1972; Kumari and Nair 1983). Comparable treatments in the unirradiated plasmodia lead to several hours of delay towards mitosis, followed by abnormal (abortive) mitosis in a majority of the nuclei (Devi *et*

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Abbreviations used: PIM, Postirradiation mitosis; SDM, semi-defined medium; PFM, post-fusion mitosis.

al 1968b; Guttes *et al* 1969), such that no further mitosis occurred in the presence of the drug (Kumari and Nair 1983). The complete actinomycin resistance of the post-irradiation mitotic cycle in this system is also accompanied by a reduction in the activity of this drug on the rate of overall RNA synthesis (Dworzak and Sachsenmaier 1973; Sachsenmaier and Dworzak 1976). Cellular level autoradiographic studies also confirmed this (Kumari and Nair 1983). In another study, we had subjected ^{3}H-uridine pulse-labelled RNA from the irradiated plasmodia to sucrose density gradient centrifugation. This showed that the irradiated plasmodia synthesize preferentially low molecular weight RNA types, including 4S RNA, and that there is an overall reduction in the rate of synthesis of rRNA (Kumari and Nair 1981). As a follow-up of this, we have now analysed the actinomycin sensitivity of the RNA synthesized by the irradiated plasmodia before and after the first PIM, employing essentially similar methods for isolation and characterization of RNA as Kumari and Nair (1981). It is found that while all the density classes of RNA synthesized during the delay period prior to the first PIM are susceptible to actinomycin as much as the control system, complete resistance was seen in the case of RNA synthesized during the cycle after the first PIM. The results of this study are discussed here.

2. Materials and methods

2.1 *Culturing of the organism and determination of mitotic stages*

The Wisconsin strain (M3C) of *Physarum polycephalum* was grown as microplasmodia in shaken cultures on a semi-defined medium (SDM) (Daniel and Baldwin 1964), at a temperature of 24°C. Mitotically synchronous surface (macro) plasmodia were made by the coalescence of pooled microplasmodial suspension (0·4 ml aliquots) on Whatman No. 40 filter paper (Guttes and Guttes 1964). A set of macroplasmodia made from pooled microplasmodial suspension shows synchronous mitosis among themselves and hence are referred to as sister plasmodia. Such a set of sister macroplasmodia in G2-phase, prior to the third post-fusion mitosis (PFM), was used for the irradiation experiments. The mitotic timings were determined by observation of ethanol-fixed smear preparations made from explants of plasmodia removed at different times in the mitotic cycle, under the oil immersion lens of a phase microscope (Guttes *et al* 1961).

2·2 *Irradiation, actinomycin treatment and labelling of RNA*

The schedule for irradiation, actinomycin D treatment, and ^{3}H-uridine labelling, in relation to the mitotic cycle is given in figure 1.

For irradiation, a Philips 15-watt germicidal lamp was employed, which emits approximately 90% of its UV energy at 2537 Å, at a dose rate of 10·26 $Jm^{-2}s^{-1}$. Total dose used in our experiments was 1400 Jm^{-2}.

Actinomycin D (Boehringer) was used at a concentration of 200 μg ml^{-1} of SDM. It is known from earlier studies that, in *Physarum,* actinomycin D is required to be used at this fairly high concentration for any significant biological effect, in *in vivo* experiments (Mittermayer *et al* 1965; Kumari and Nair 1983). The duration of pretreatment with the drug, prior to ^{3}H-uridine labelling [80 μCi $ml^{-1}$of SDM

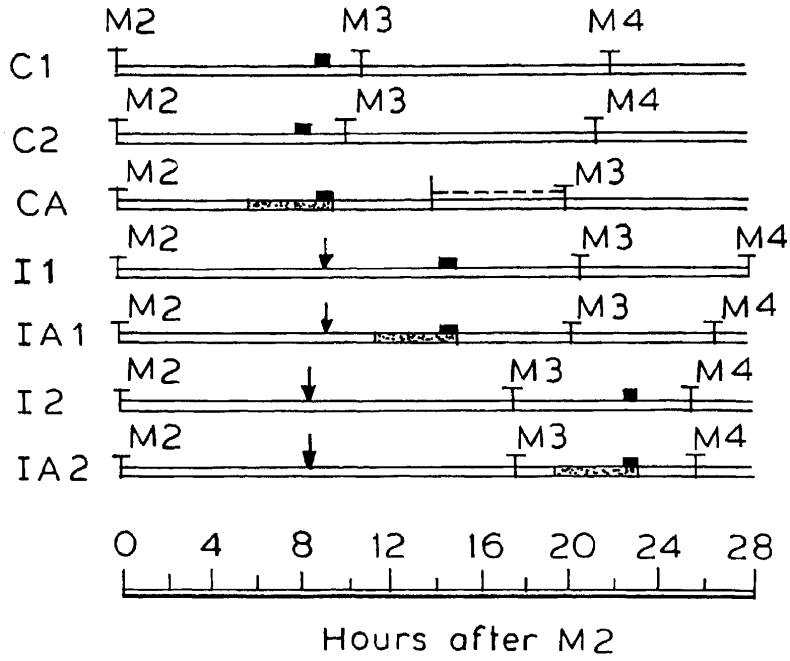


Figure 1. Schedule of irradiation, actinomycin treatment and ^{3}H-uridine labelling.

C1 and C2, Control plasmodia; RNA analysis data for C1 given in figure 2; CA, control Plasmodium, treated with actinomycin, RNA analysis data given in figure 3; I1 and I2, irradiated plasmodia, RNA analysis data given in figures 4 and 6 respectively; IA1 and IA2, irradiated plasmodia, treated with actinomycin, RNA analysis data given in figures 5 and 7 respectively. The schedule for irradiation, actinomycin treatment, and ^{3}H-uridine labelling for plasmodia, whose RNA analysis data are given in figures 8 and 9 are same as those for figures 6 and 7, except that the ^{3}H-uridine pulse duration was 15 min. This schedule refers to the data given in figures 2-9 and table 1.

M2, Second PFM; M3, third PFM in the case of control and first PIM in the case of irradiated plasmodia. ↓, Time of UV-irradiation (1,400 Jm^{-2}); ⁘, actinomycin (200 μg ml^{-1} of SDM) treatment for 3 h 30 min ■ ^{3}H-uridine (80 μCi/ml) pulse for 30 min. In the case of actinomycin treated plasmodia, the labelling was carried out during the last 30 min of drug treatment. ⫶, Duration of prolonged, arrested mitosis with condensation of chromatin as in metaphase, but with abnormal spindle.

(Bhabha Atomic Research Centre, Bombay); sp. activity 14 Ci/mmol], in all cases was 3 h. Labelling was also carried out in the presence of the drug. The duration of labelling was 30 min in most of our experiments, except in a few, where it was 15 min. Details are given in the figure legends.

2.3 *Extraction and fractionation of RNA*

RNA was extracted by the sodium dodecyl sulphate (SDS)-phenol-diethylpyrocarbonate method of Zellweger and Braun (1971). Each Plasmodium was shaken on a vortex for 5 min at 4°C in a mixture of 2·85 ml of a buffer containing 0·5 M sodium chloride, 0·02 M sodium citrate, 0·01 M Tris, pH 7·4 (buffer I), 0·15 ml 20% SDS, and 0·2 ml diethylpyrocarbonate. Diethylpyrocarbonate was used here as an inhibitor of ribonuclease (Solymosy *et al* 1968). The above suspension was then

Table 1. A quantitative analysis of the RNA fractions in sucrose gradients, the sedimentation pattern of which are given in figures 2–7.

Sample	Peak regions counted from bottom of gradient	Absorbance/region[a]	dpm/region[b]	dpm/absorbance[c]	Inhibition due to actinomycin/irradiation or both based on unirradiated controls[d] (%)		Inhibition or increase (↑) due to actinomycin calculated with respect to irradiated system (IA 1 compared to I1 and IA2 compared to I2) (%)
					C1	C2	
C1	I	5·15	8,230	1600	—	—	—
(unirradiated	II	3·78	11,440	3030	—	—	—
control 1)	III	1·55	6,190	3990	—	—	—
				(1·9; 2·5; 1·7)			
C2	I	6·38	9,520	1490	—	—	—
(unirradiated	II	2·76	5,600	2030	—	—	—
control 2)	III	1·64	4,140	2520	—	—	—
				(1·4; 1·7; 1·4)			
CA	I	4·84	1,650	340	78·75	77·18	—
(unirradiated	II	2·61	1,620	620	79·54	69·46	—
-actinomycin	III	0·84	1,120	1330	66·67	47·22	—
treated)				(1·8; 3·9; 2·8)			
I1	I	5·75	1,610	280	82·50	81·21	—
(irradiated,	II	2·74	1,180	430	85·81	78·82	—
labelled prior	III	1·35	1,850	1370	65·66	45·63	—
to first PIM)				(1·5, 4·9, 3·9)			

IA1	I	5·04	340	70	95·62	95·30		75·00
(same as I1, but	II	2·40	260	110	96·37	94·58		74·42
treated with	III	1·40	570	410	89·72	83·73		70·07
actinomycin)				(1·6; 5·9; 4·6)				
I2	I	5·27	3,590	680	57·50	54·36		—
(irradiated, labelled	II	2·66	2,940	1110	63·37	45·32		—
prior to second	III	1·44	1,840	1280	67·92	49·21		—
PIM)				(1·6; 1·9; 1·4)				
IA2	I	5·57	4,910	880	45·00	40·94	(↑)	29·41
(same as I2,	II	2·30	2,810	1220	59·75	39·90	(↑)	9·91
but treated with	III	0·85	1,690	1990	50·13	21·03	(↑)	55·47
actinomycin)				(1·4; 2·3; 1·9)				

[a]Total absorbancy at 260 nm for all the fractions in the three regions represented by the respective peaks.

[b]Total dpm for all the fractions in the above three regions.

[c]Total dpm for each region/total absorbance for the corresponding region.

The numbers in parentheses are II/I-ratio of dpm/absorbance of region II with respect to the dpm/absorbance of region I; III/I-ratio of dpm/absorbance of region III to the dpm/absorbance of region I; III/(I + II)/2- ratio of dpm/absorbance of region III to the dpm/absorbance of region (I + II/2).

[d]Two controls (C1 and C2) are given here to show the possible variation in ^{3}H-uridine incorporation rate between two unperturbed sister plasmodia.

extracted twice with 3 ml phenol at 60°C and twice with 3 ml phenol at 4°C, each time adding 0·2 ml diethylpyrocarbonate to the aqueous layer. RNA was precipitated from the aqueous layer of the final extract with two volumes of cold ethanol (–20°C), containing 2% sodium acetate. The precipitate was collected by centrifugation for 45 min at 10,000 rpm (12,000 *g)* in the SW 50·1 rotor of Beckman L8-55M ultracentrifuge. This was then dissolved in 1 ml of a buffer containing 0·1 M sodium chloride, 0·1 mM magnesium chloride, 0·1 M sodium acetate, pH 5·5 (buffer II) and was precipitated, as above, once more and stored under ethanol at – 20°C.

RNA thus extracted was fractionated on a linear 5–30% (w/v) sucrose gradient. The sucrose solutions were made using buffer II. The RNA (0·4 ml) dissolved in buffer II was layered on top of a 4·6 ml gradient. Gradients were centrifuged at 45,000 rpm (243,000 *g*) in a Beckman SW 50·1 rotor for 3 h 30 min at 4°C. Fractions of 10 drops each were collected in tubes. To each tube, 1 ml of distilled water was added and the absorbancy was measured at 260 nm, using a Beckman DU-6 spectrophotometer. The sedimentation coefficients of the stable RNA types in *Physarum,* which include the two types of ribosomal RNA and the tRNA, were taken to be 26S, 19S and 4S, respectively and are based on earlier studies (Melera *et al* 1970; Zellweger and Braun 1971; Grant 1973).

2.4 *Assay of radioactivity*

For radioactivity assay, aliquots from each of the above samples were spotted on Whatman GF/C glass fibre filters and counted in a LKB RackBeta –1215 LSC, using a toluene, PPO, POPOP cocktail.

2.5 *Quantitative evaluation of the gradients*

A quantitative evaluation of the RNA fraction in the various sucrose gradients is given in table 1. For this, the fractions in a gradient have been grouped into three regions, starting from the bottom. Peaks representing the three stable classes of RNA, namely, 26S, 19S, and 4S have been taken as markers for this categorization i.e., region I represented by 26S, region II by 19S and region III by 4S. The absorbance/region and dpm/region were calculated separately and from this the dpm/absorbance for each of these regions was obtained. The ratio of the dpm/absorbance of regions II and III in terms of region I taken as a standard is also provided. Percentage inhibition of RNA synthesis due to actinomycin and that due to irradiation is also given as part of the quantitative evaluation.

3. Results

In the case of the unirradiated, control plasmodia, even at the fairly high concentration (200 μg ml^{-1}) used by us, actinomycin has a preferential inhibitory action on rRNA synthesis (both 26S and 19S types), when compared to the low molecular weight types, including the 4S category (figures 2 and 3 and table 1). It is also seen that irradiation alone inhibited rRNA synthesis, even more than that due to actinomycin. In the low molecular weight region of the gradient, the inhibition by actinomycin or UV is about the same (compare figure 2 with figures 3 and 4 and refer table 1). However, as UV inhibits both rRNA types more than actinomycin,

on a comparative basis, in an irradiated system, there is greater synthesis of low molecular weight RNAs when compared to rRNA (figure 4).

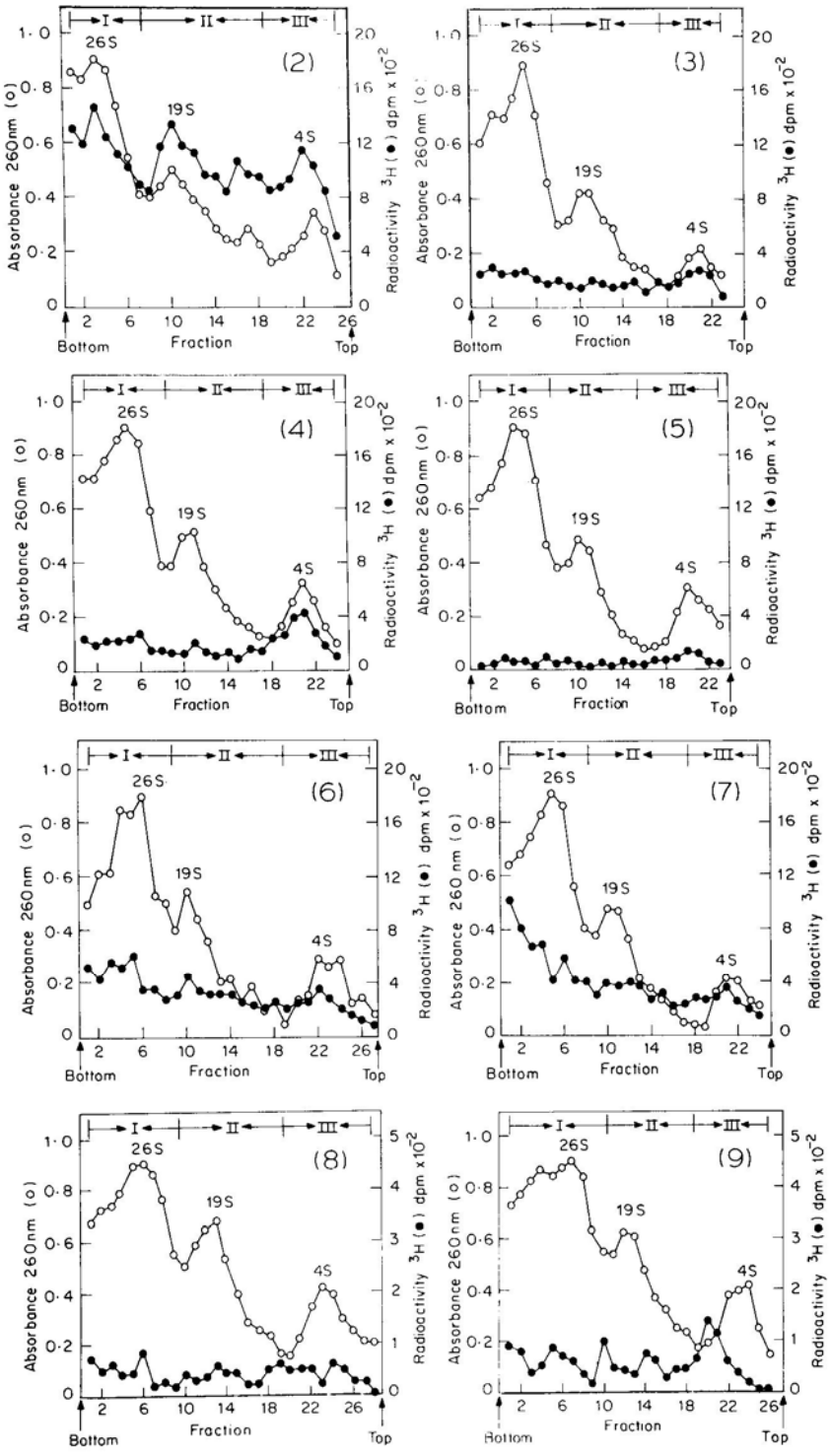
Total inhibition due to irradiation and actinomycin treatment of all classes of RNA isolated during the UV-induced, extended G2-phase prior to the first post-irradiation mitosis is more than that due to actinomycin alone in the unirradiated system (compare type IA1 with CA in table 1). In other words, some additive effect of irradiation and actinomycin on RNA synthesis is seen during this period. Also, at this time, the extent of sensitivity of the irradiated system to actinomycin is not different from that shown by the control (figures 2 and 3 compared with figures 4 and 5). On an average, more than 70% inhibition of synthesis of the various sedimentation classes of RNA is seen in the irradiated, actinomycin-treated Plasmodia in comparison with the corresponding irradiated plasmodia (compare type 1A1 with I1 in table 1). About the same percentage inhibitions are also induced by actinomycin in the case of control, unirradiated plasmodia (type CA compared to types C1 or C2 in table 1). In fact, in the low molecular weight region, the percentage inhibition induced by actinomycin in the irradiated plasmodia is even more than that in the control (types Ia1 and I1 in table 1 compared to CA and C1 or C2).

The characteristics of the RNA synthesized in the irradiated Plasmodium after the first postirradiation mitosis is very different, when compared to the above described synthesis during the delay period prior to that mitosis. Although there is still substantial inhibition of transcription when compared to controls, the rate increased after the first PIM. What is perhaps more striking is the total actinomycin insensitivity of all the sedimentation classes of RNA synthesized during this period. In fact, the synthetic activity is greater in the drug-treated, irradiated Plasmodium (figures 6 and 7 and table 1). The higher synthetic activity of the drug-treated category can also be shown after shorter ^{3}H-uridine pulses (figures 8 and 9). The comparison here is made with respect to the corresponding irradiated plasmodium. Percentage-wise, the increased synthesis is greater in the low molecular weight region of the gradient.

4. Discussion

In confirmation of the results of earlier studies, it is observed that UV induces severe repression of overall transcription in *P. polycephalum* (Kumari and Nair 1984), a major part of which is accounted for by rRNA (Kumari and Nair 1981). In fact, it is seen that the repression of transcription continues throughout the extended G2-phase prior to the first PIM (figure 4), and also during the following mitotic cycle (figure 6). However, the present studies show, in addition, that the rate of overall transcription, particularly in the higher molecular weight category, increased following the first PIM in comparison to that before it, although still being significantly less than that in the control.

The repression particularly of rRNA is understandable in view of the proposed feedback nature of the control of this RNA synthesis in our system (Kumari and Nair 1981). The feedback here is necessitated because of the already described, UV-induced degeneration of a large number of nuclei and the resultant alteration of the nucleo-cytoplasmic ratio in this syncytial system. Because of this, the total cellular


(2)
(3)
(4)
(5)
(6)
(7)
(8)
(9)
26S
19S
4S
I
II
III
Absorbance 260nm (o)
Radioactivity ^3H (●) dpm x 10^{-2}
Bottom
Fraction
Top

Figures 2-9.

RNA/DNA and protein/DNA ratios, at the time of the first delayed mitosis after radiation are much larger than that seen in the control at the time of the corresponding undelayed mitosis (Devi *et al* 1968a). Since much of the RNA here, just as in any cellular systems, is accounted for by rRNA, the repression of rRNA synthesis to a great extent, helps in bringing down, the above, increased ratios back to the control level.

It is known that the sensitivity of the *P. polycephalum* plasmodia, with respect to mitotic delay induced by actinomycin, decreased rapidly after UV-irradiation, such that, following the first PIM, the plasmodia remained insensitive to continuous actinomycin exposure for a few mitotic cycles (Devi and Guttes 1972; Kumari and Nair 1983). In view of this, the present demonstration of completely actinomycin-insensitive, heterogenously sedimenting RNA synthesis during the first post-irradiation mitotic cycle assumes importance. Moreover, an enhanced synthesis, particularly in the low molecular weight region of the gradient, was also seen in the drug-treated plasmodia (compare figure 6 with figure 7 and figure 8 with figure 9).

There have been many reports of lower sensitivity or even increased synthesis of some RNA types in the presence of actinomycin in different cellular systems. In L cells treated with low concentrations of actinomycin, synthesis of 28S and 18S RNA can be inhibited without a corresponding decrease in the 5S RNA that accumulates in the nucleoplasm (Perry and Kelley 1968). An increase in tyrosine aminotransferase synthesis, following the addition of actinomycin to steroid-hormone-induced hepatoma cell cultures, led Tomkins *et al* (1969) to propose that steroids inactivate an unstable-mRNA specific, suppressor protein, thus increasing the availability of the mRNA for the above aminotransferase. In Tomkin's system, in the presence of actinomycin, no further synthesis of the unstable mRNA coding for the suppressor protein will occur and hence the superinduction of the aminotransferase in its

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Figures 2-9. Sucrose density gradient analysis of RNA from plasmodia.

RNA was labelled with 80 μCi/ml of ^{3}H-uridine as per schedule given in figure 1. Abscissa: Fraction number; Ordinate: Absorption at 260 nm (left) (O); Radioactivity (^{3}H) in dpm (right) (●). For easy comparison of the data from the different gradients, both the absorbance and the radioactivity are uniformly scaled down, such that the maximum absorbance for 26S is 0·9. I, II and III in each figure indicate the regions covered by the RNA peaks, 26S, 19S and 4S respectively. The arrows mark the limits of the respective peaks and indicate the fractions used for calculations of total absorbance and total radioactivity for each region as given in table 1. **2**. Unirradiated Plasmodium labelled for 30 min, 2 h prior to the third PFM. **3.** Unirradiated Plasmodium pretreated for 3 h with actinomycin and labelled for 30 min in the presence of the drug. Treatment with the drug started 5 h prior to the third PFM, such that labelling could be carried out along with the control given in **2. 4.** Plasmodium irradiated 1 h 40 min before the third PFM and labelled for 30 min, 5 h after irradiation, during the delay period prior to the first PIM (i.e., the delayed third PFM). **5.** Similar Plasmodium as in **4,** but pretreated with actinomycin for 3 h and labelled for 30 min in the presence of the drug. Treatment with actinomycin started *2* h after irradiation, such that labelling could be done with the Plasmodium given in **4. 6.** Plasmodium irradiated 1 h 40 min before third PFM and labelled for 30 min, 5 h after the first PIM (i.e., during the first postirradiation mitotic cycle). **7.** Similar Plasmodium as in **6**, but pretreated for 3 h with actinomycin and labelled for 30 min in the presence of the drug. Treatment with the drug started 2 h after the first PIM, such that labelling could be done with the Plasmodium given in **6. 8.** Same as **6,** except that the pulse-labelling with ^{3}H-uridine was for 15 min. **9.** Same as **7**, except that the labelling with ^{3}H-uridine was carried out for 15 min.

presence. With respect to superinduction, the postirradiation mitotic cycle in *Physarum* appears to be analogous to the above hormone-induced cell cultures. It has been reported that nucleolar 4–7S RNA is increased after treatment of experimental animals with actinomycin (Busch and Smetana 1970). Actinomycin has also been used to amplify the labelling of mitochondrial RNA *in situ* (Borst and Grivell 1971). Benecke and Penman (1977) also reported the presence of a new class of small nuclear RNAs of discrete sizes between 6S and 10S in HeLa cells, which are not affected by a certain concentration of actinomycin to which rRNA synthesis is susceptible. They observed that this class of RNA which is apparently associated with the chromatin and nuclear matrix, is also resistant to rather high concentrations of α-amanitin.

It is known that rRNA synthesis in *P. polycephalum* is preferentially susceptible to the action of actinomycin (Mittermayer *et al* 1964; Grant 1972); just as in the case of other eukaryotes (Penman *et al* 1968). Therefore, the resistant synthesis seen in our system in all probability must represent some other class of RNA. Also, the resistant synthesis is seen at a time when the rate of RNA synthesis in general has increased (figure 6 and table 1), over its severe repression during the delay period prior to the first PIM (figure 4 and table 1). In this connection it is pertinent to point out that both in the irradiated system prior to the first PIM and in the control, approximately 20% of the heterogenously sedimenting synthetic activity was found not to be susceptible to the fairly high concentration of the drug (200 μg ml^{-1}) used by us in the present studies. It would then appear that, following the first PIM, the predominant synthetic activity is of the latter type. If so, the post-irradiation mitotic cycle would be an ideal system for the further characterization of this RNA(s) showing a fairly high resistance to actinomycin, especially because of the observed increased synthesis in the presence of the drug. The heterogenously sedimenting synthetic activity here, with increased activity in the low-molecular weight region, may represent a single species of RNA and its precursors or more than one kind of RNA. It would also be interesting to investigate the relationship, if any, of this RNA(s) to the reported actinomycin insensitivity of the mitotic process and the duration of the mitotic cycle in this system. It is perhaps relevant here to note that the duration of the fairly short, postirradiation mitotic cycle in *P. polycephalum* can be further shortened by continuous actinomycin exposure, starting a few hours before the onset of this mitotic cycle (Devi and Guttes 1972; Kumari and Nair 1983).

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