RNA Synthesis in a Cell Cycle-Specific Temperature Sensitive Mutant from a Hamster Cell Line†

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ABSTRACT: tsAF8 cells, a temperature-sensitive mutant of BHK cells, grow normally at 32 °C but are arrested at 39.5–40 °C at a point in the cell cycle located in mid-G_1. When confluent monolayers of tsAF8 cells are stimulated to proliferate by serum at the permissive temperature, they enter DNA synthesis between 20 and 30 h after stimulation, while no increase in DNA synthesis occurs in cells stimulated at 40 °C. At the permissive temperature, both nuclear and nucleolar RNA synthesis, as determined in vitro in isolated nuclei or nucleoli, increase until the cells are well advanced into S phase. Maximum peaks between 20 and 24 h were twofold and fourfold above stimulated controls for nuclear and nucleolar RNA synthesis, respectively. At the nonpermissive temperature, nuclear and nucleolar RNA synthesis increase normally until 16-h after stimulation. Thereafter, nuclear RNA synthesis declines rapidly (at 22 h, it is at the level of unstimulated controls), while nucleolar RNA synthesis continues at a high level (still above the control level at 30 h). The decrease in nuclear RNA synthesis at the nonpermissive temperature is due to a decrease in α-amanitin sensitive RNA synthesis. The in vitro nuclear activity of RNA polymerase II, in tsAF8 cells at 40 °C, decreases to 50% of control level of 24 h, and by 48 h it is virtually nonexistent. The results indicate that RNA polymerase II activity is required for the entry of tsAF8 cells into S. In addition, these cells constitute a useful model to study the relationship between ribosomal and nonribosomal RNA synthesis.

The tsAF8 cells are a temperature-sensitive (ts) mutant of BHK cells that were originally isolated by Basilico and coworkers (Meiss & Basilico, 1972; Burstin et al., 1974). These cells grow normally at 32–34 °C but become blocked in the G_1 phase of the cell cycle at the nonpermissive temperature, 39.5–40 °C (Burstin et al., 1974). The ts block has been located in mid-G_1 at a point situated 6–8 h before the onset of DNA replication (Burstin et al., 1974; Kane et al., 1976). The ts function is necessary for the replication of adenovirus 2 (Nishimoto et al., 1975) and the mid-G_1 block is released in somatic cell hybrids between tsAF8 and SV-40 transformed human fibroblasts, by the presence of human chromosome 3 (Ming et al., 1976).

Because an increase in RNA synthesis, especially rRNA synthesis, is a striking characteristic of resting cells stimulated to proliferate (see Discussion), we have investigated nuclear and nucleolar RNA synthesis in quiescent tsAF8 cells stimulated by serum at both the permissive and the nonpermissive temperature.

Methods and Materials

Cell Cultures. AF8, a temperature-sensitive mutant of BHK 21/13 cells, was grown in Dulbecco's high glucose medium (GIBCO) plus 10% donor calf serum and antibiotics, as previously described (Meiss & Basilico, 1972; Burstin et al., 1974; Kane et al., 1976). Quiescent cultures were produced by allowing cells to grow to apparent confluency (5–6 days) and then decrease the serum concentration to 0.5% for 48 h. These nongrowing cells were then stimulated to proliferate by replacing the serum-deficient medium with fresh medium containing 10% serum. The parent cell line, BHK cells, was also grown in Dulbecco's medium as AF8 cells (see above).

Isolation of Nuclei. Nuclei free of cytoplasmic contamination were obtained by homogenization with a glass Dounce homogenizer of cells in a buffer containing 10 mM Tris-HCl (pH 8.0), 2 mM $Mg(Ac)_2$, 3 mM $CaCl_2$, 1 mM $PhCH_2SO_2F$,[1] to which Triton X-100 and sucrose were then added to a final concentration respectively of 0.5% and 0.33 M. The nuclei were then centrifuged at 2200 rpm in an International PR-6 centrifuge, resuspended in the same buffer and centrifuged as above. The final nuclear pellet was resuspended in TGMED (Marzluff et al., 1973).

Isolation of Nucleoli. The method used for isolation of nucleoli was essentially that described by Muramatsu et al. (1974) with slight modifications. The technique and the criteria used to determine the purity of the nucleoli have been described in detail in a previous paper from this laboratory (Huang & Baserga, 1976).

Determination of DNA Specific Activity. The specific activity of DNA was determined after a 1 h pulse of [^{3}H]thymidine (1 μCi/mL) by dividing the acid-insoluble radioactivity by the amount of DNA, determined by the procedure of Burton (1968).

Nuclear Assay for RNA Synthesis. The incubation mixture was that of Marzluff et al. (1973) and contained in a final volume of 125 μL: glycerol 12.5%, magnesium acetate (5 mM), MnCl (0.75 mM), Tris-HCl (37.5 mM), pH 8.0, 0.0625 mM NaEDTA, 1.0 mM dithiothreitol, KCl (see below), 0.3 mM ATP, GTP, CTP, 0.04 mM UTP plus 2.5 μCi/assay of [^{3}H]UTP and nuclei (20–30 μg of DNA). The radioactivity incorporated into acid-precipitable material was determined as described by Marzluff et al. (1973). Contrary to the report of Marzluff et al. (1973), who used myeloma cells, and of Ernest et al. (1976), with hen oviduct nuclei, AF8 nuclei do better at 37 °C than at 25 °C. Accordingly our incubations were carried out at 37 °C.

The percentage of α-amanitin resistant RNA synthesis in isolated nuclei was determined at various concentrations of

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[1] Abbreviation used: $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride.

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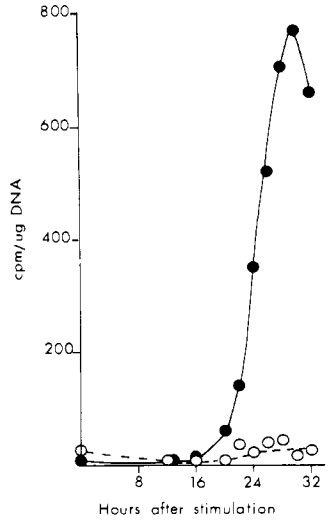

FIGURE 1: Stimulation of DNA synthesis in confluent monolayers of tsAF8 cells. Serum-deprived monolayers of cells were stimulated with fresh medium containing 10% serum at zero time. The cells were pulsed with [^{3}H]thymidine 1 h before the times indicated on the abscissa, and the acid-insoluble radioactivity was determined, as described in Methods and Materials. (●—●) Cells stimulated at 32 °C; (○- - -○) cells stimulated at 40 °C.

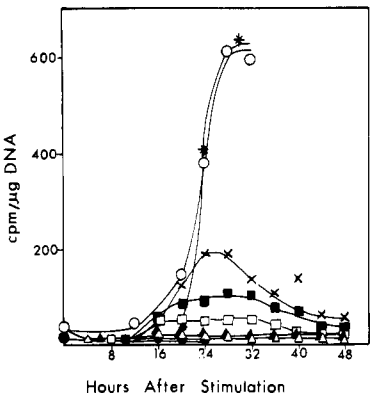

FIGURE 2: DNA synthesis in tsAF8 cells shifted up at various times after serum stimulation. AF8 cells were stimulated as described in Figure 1 and shifted to 40 °C at various times after stimulation, as follows: (△) shifted-up at 4 h; (▲) at 6 h; (□) at 8 h; (■) at 10 h; (X) at 12 h; (✱) at 20 h; (○) no shift-up. (●) Shifted up at zero time. DNA synthesis was determined as in Figure 1.

KCl. Confirming the results of Marzluff et al. (1973) nuclear RNA synthesis remains at the same level at KCl concentrations between 0.15 and 0.30 M. However, the percentage of α-amanitin resistant activity decreases with increasing KCl concentration from 68% at 0.15 M to 66% at 0.25 M, to 37% at 0.35 M. In all these experiments, because the concentration of α-amanitin was 1.0 μg/mL, the fraction of α-amanitin sensitive activity represents essentially RNA polymerase II activity (Chesterton & Butterworth, 1971; Chambon, 1975). We have selected a concentration of 0.35 M KCl as the most suitable for these experiments. At this concentration of KCl, AF8 nuclei remain intact. Indeed AF8 nuclei are not broken by KCl concentration up to 1.0 M, obviously at variance with nuclei from other cells (Marzluff et al., 1973; Lindell, 1975).

The requirements for RNA synthesis by isolated nuclei (dependence on all four ribonucleoside triphosphates, linearity with nuclear concentration, etc.) have already been detailed in previous papers from this and other laboratories (Marzluff et al., 1973; Ernest et al., 1976; Kane et al., 1976). We shall only mention that the addition of a proteolytic inhibitor, such as phenylmethanesulfonyl fluoride (1.0 mM), had no effect on nuclear RNA synthesis (not shown) confirming the findings of Lindell (1975). Since these experiments were carried out over a period of 1 year, the absolute values changed with the condition of the cells, the specific activity of UTP, etc., and for this reason, in some cases, we are expressing the values for RNA synthesis in percent of control values.

Nucleolar Assay. Nucleolar RNA synthesis was assayed in the same incubation mixture used for the nuclei, which is not much different from the incubation mixtures used for nucleoli by other investigators (Nicolette & Babler, 1974; Lindell, 1975; Grummt, 1975). Under these conditions, nucleolar RNA synthesis is completely resistant to α-amanitin at 1.0 μg/mL, even when the concentration of KCl is raised to 0.8 M (Huang & Baserga, 1976), and is not affected by proteolytic inhibitors (not shown). The cofactor's requirements for our nucleolar preparations were essentially the same as described by other investigators (Villalobos et al., 1964; Lindell, 1975; Ferencz & Seifart, 1975).

Materials. [^{3}H]Thymidine (6.7 Ci/mmol) and [^{3}H]UTP (varying in specific activity from 22 to 27.6 Ci/mmol) were purchased from New England Nuclear Co. α-Amanitin was obtained from Calbiochem (La Jolla, Calif.).

Results

Stimulation of Cell Proliferation. Monolayers of tsAF8 cells were made quiescent by 2 days in 0.5% serum at the permissive temperature and were stimulated by changing to fresh medium plus 10% serum. Figure 1 shows the extent of DNA synthesis in unstimulated controls and in cells stimulated at either the permissive or the nonpermissive temperatures. The time course is similar to that reported by Burstin et al. (1974) with a broad slope of entry into S (at 32 °C), and a peak at 28–32 h. At 40 °C there is no appreciable increase in DNA synthesis. During the same period of time, the amount of DNA per culture dish almost doubles in cells stimulated at 32 °C, while it remains unchanged in cells stimulated at 40 °C (not shown). The data of Figure 1 refer to [^{3}H]thymidine incorporated into DNA, but essentially the same curve is obtained if the percentage of cells in DNA synthesis is determined by autoradiography. After 48 h in 0.5% of serum, less than 5% of the AF8 cells can be labeled by exposure to [^{3}H]thymidine for 24 h. About 80% of the cells enter DNA synthesis after stimulation at 32 °C. At the nonpermissive temperature the cells remain viable for at least 48 h, but fewer than 10% enter DNA synthesis (unpublished data). The parent cell line (BHK cells) is stimulated equally well at 34 and 40 °C (not shown).

Shift-Up Experiments. Quiescent AF8 cells were stimulated with 10% serum at 32 °C and shifted-up to 40 °C at various times after stimulation (Figure 2). When cells were shifted-up 20 h after stimulation, they entered DNA synthesis as stimulated cells kept all the time at 32 °C. Entry into DNA synthesis was completely inhibited when the cells were shifted up 4–6 h after stimulation. Intermediate degrees of inhibition were obtained when cells were shifted up between 8 and 16 h after stimulation.

Shift-Down Experiments. Quiescent AF8 cells were stimulated at 40 °C with 10% serum and shifted down to 32 °C at various times after stimulation (Figure 3). In every case the entry into DNA synthesis of shifted-down cells was delayed by a time roughly equal to the time spent at 40 °C. Thus, cells shifted down 6 h after stimulation entered DNA synthesis 5 h later than cells always kept at 32 °C, while, in cells shifted down 10 h after stimulation, the delay was 11 h. For clarity

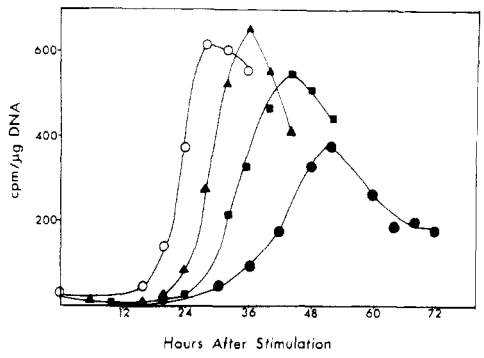

FIGURE 3: DNA synthesis in tsAF8 cells shifted down at various times after serum stimulation. Same condition as in Figures 1 and 2, except the cells were stimulated at 40 °C and shifted down at: zero time (O—O); 6 h (▲—▲); 10 h (■—■); 20 h (O—O).

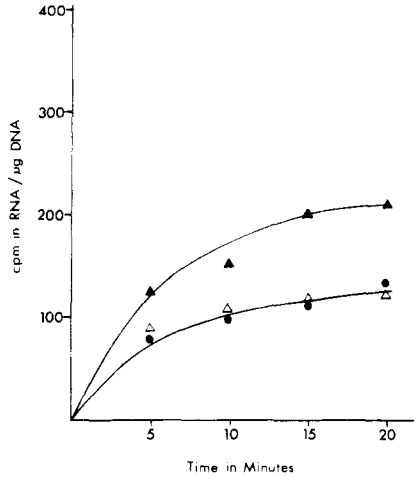

FIGURE 4: Time curve of RNA synthesis in nuclei isolated from tsAF8 cells. The nuclear assay is described in Methods and Materials. The abscissa gives the incubation time. (●—●) Unstimulated controls; (△—△) stimulated for 26 h at 40 °C; (▲—▲) stimulated for 26 h at 32 °C.

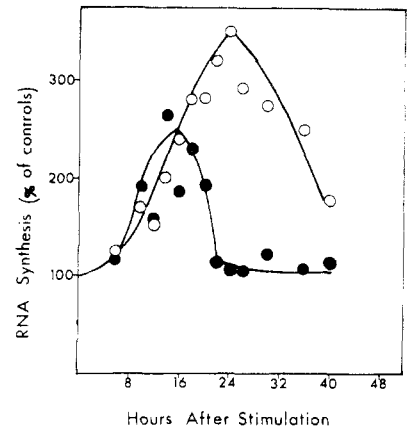

FIGURE 5: Nuclear RNA synthesis in serum-stimulated confluent monolayers of tsAF8 cells. Resting cells were stimulated to proliferate as described in Figure 1, and RNA synthesis was determined in isolated nuclei as described in the text. (O—O) Cells stimulated at 32 °C: (●—●) cells stimulated at 40 °C. Each point is the average of at least two experiments, but the points between 16 and 30 h and the controls were repeated more than ten times.

TABLE I: Amount of α-Amanitin Resistant or Sensitive Nuclear RNA Synthesis in tsAF8 Cells Stimulated at either 32 or 40 °C.[a]

Time after stimulation	cpm in RNA/μg of DNA		
	Total	α-Amanitin resistant	α-Amanitin sensitive
controls (32 or 40 °C)	200	68	132
stimulated 12 h, 32 °C	270	123	147
stimulated 12 h, 40 °C	267	165	102
stimulated 18 h, 32 °C	400	231	169
stimulated 18 h, 40 °C	277	197	80
stimulated 24 h, 32 °C	363	184	179
stimulated 24 h, 40 °C	204	134	70

[a] Stimulation and harvesting of cells and isolation and incubation of nuclei were carried out as described in Figures 5 and 7. The amount of α-amanitin in the incubation mixture was 1.0 μg/mL. Each point is the average of at least two experiments, but in most cases the experiments were repeated 3–5 times. Variations in duplicate samples were ±10% of the averages.

only four curves are shown in Figure 3, but several intermediate shift-down points were also investigated, with the consistent result of a delayed entry into DNA synthesis, the delay being equal to the time spent at 40 °C.

Nuclear RNA Synthesis. The term RNA synthesis is used in this paper in the same way as used by Marzluff et al. (1973), i.e., to indicate the incorporation of [^{3}H]UTP into acid-precipitable counts by isolated nuclei under the conditions of incubation described above. For essentially similar conditions, Reeder & Roeder (1972) and Gross & Pogo (1976) also used the term RNA synthesis, but other investigators have preferred the term "endogenous RNA polymerase activity" (Lindell, 1975; Weinmann et al., 1976; Hardin et al., 1976). Since either term seems to be acceptable, we have chosen RNA synthesis for its convenient brevity. Figure 4 shows the time course of RNA synthesis by isolated AF8 nuclei incubated at 37 °C. Unless otherwise specified (see below), the values for RNA synthesis in subsequent experiments are those obtained after 15 min of incubation at 37 °C.

Figure 5 shows the effect of serum stimulation on nuclear RNA synthesis in tsAF8 cells stimulated at either 32 or 40 °C. After stimulation at 32 °C there is a modest but prompt increase between 0.5 and 3.0 h, about 40% above control levels (which is not shown in Figure 5), followed by a gradual increase to a maximum (300% above controls) between 18 and 24 h. At 40 °C, nuclear RNA synthesis increases up to 14–16 h after stimulation, then it decreases rapidly, reaching the level of unstimulated controls by 22 h. After the 28th hour, nuclear RNA synthesis decreases also at the permissive temperature, but even at 40 h it is higher than in unstimulated controls and higher than in cells stimulated at 40 °C.

Effect of α-Amanitin on Nuclear RNA Synthesis. In resting cells the percentage of α-amanitin resistant nuclear RNA synthesis is 35–40%, in agreement with the results of Reeder & Roeder (1972). Table I shows the variation in α-amanitin-resistant nuclear RNA synthesis at selected times after stimulation, at either temperature (32 or 40 °C). In this table the actual cpm of [^{3}H]UTP incorporated into RNA (in the presence or absence of α-amanitin) are given, and the α-amanitin sensitive cpm are calculated by subtraction. Two major conclusions can be obtained from Table I: (1) stimulation of cell proliferation causes an increase in α-amanitin resistant RNA synthesis; and (2) stimulation to proliferate causes only a modest increase in α-amanitin-sensitive RNA synthesis. Thus, at 18 h the amount of α-amanitin-resistant RNA synthesis is 3.5 times the control level, while the α-amanitin-sensitive amount has increased only 25%. At the same time after stimulation, but at nonpermissive temperature of 40 °C, the amount of α-amanitin-sensitive RNA synthesis is actually lower than in unstimulated controls, a point of considerable importance that will be discussed below.

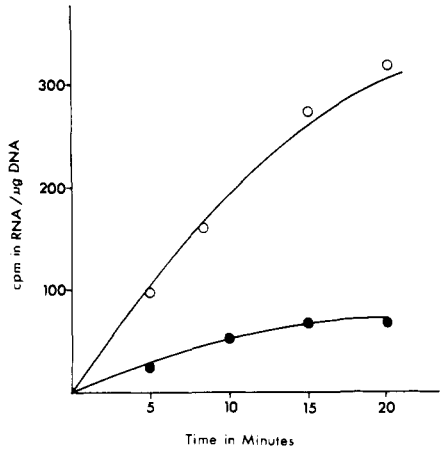

FIGURE 6: Time course of RNA synthesis in nucleoli isolated from tsAF8 cells. Nucleoli were isolated and assayed as described in Methods and Materials. The abscissa gives the incubation time in minutes. (●—●) Unstimulated controls; (○—○) cells stimulated for 18 h at 32 °C.

TABLE II: Amount of RNA Synthesis in Isolated AF8 Nuclei in the Presence of Poly[d(AT)] and Actinomycin D.[a]

	α-Amanitin resistant cpm/µg of DNA		α-Amanitin sensitive cpm/µg of DNA	
Treatment	32 °C	40 °C	32 °C	40 °C
Controls	17		35	
12 h	30	40	18	17
18 h	30	30	30	15
24 h	49	34	82	18
30 h	41	31	58	11

[a] The nuclear assay for RNA synthesis was carried out as usual except that poly[d(AT)] 25 µg/assay and actinomycin D (10 µg/assay) were added to the incubation mixture. The results are expressed in cpm/µg of nuclear DNA. The data are from a representative experiment, that was repeated several times. The first column is time after stimulation at 32 or 40 °C. All assays, though, were carried out at 37 °C.

Nucleolar RNA Synthesis. Nucleolar RNA synthesis was directly measured in isolated nucleoli, as described in Methods and Materials. Figure 6 shows the time curve of nucleolar RNA synthesis at 37 °C, which is similar to that reported by Grummt et al. (1976). α-Amanitin had no effect on nucleolar RNA synthesis. Figure 7 shows that nucleolar RNA synthesis is markedly increased in tsAF8 cells stimulated to proliferate by serum. The values given in Figure 7 are those obtained after 15 min of incubation at 37 °C. The increase is apparent at both permissive and nonpermissive temperatures and is of a magnitude reasonably in agreement with the results obtained with α-amanitin. Thus between 18 and 24-h after stimulation when α-amanitin-resistant RNA synthesis has increased 3–5 times (Table I), a fourfold increase in nucleolar RNA synthesis is also apparent (Figure 7). The most important observation in this experiment, though, is the behavior of nucleolar RNA synthesis in cells stimulated at 40 °C after the 24th hour. Nucleolar RNA synthesis remains at high levels at the nonpermissive temperature, at least for another 12 h. Again, these results are in agreement with those of Table I, showing a marked decrease in α-amanitin-sensitive RNA synthesis in cells stimulated at 40 °C for 24 h while α-amanitin-resistant RNA synthesis is much less affected.

Decay of RNA Polymerase II Activity at the Nonpermissive Temperature. Quiescent tsAF8 cells were stimulated to proliferate, as usual, at both permissive and nonpermissive temperatures and nuclear RNA synthesis determined in vitro in the presence or absence of α-amanitin (1 µg/mL), at various intervals after stimulation. Figure 8 shows that α-amanitin-resistant RNA synthesis increases after stimulation and remains high even after 40 h at 40 °C, when it is still higher than in unstimulated controls. On the contrary, α-amanitin-sensitive RNA synthesis increases only modestly after stimulation, but it steadily decreases when the cells are incubated at 40 °C, and after 48 h at the nonpermissive temperature, it is practically undetectable. This decrease in RNA polymerase II activity of AF8 cells at the nonpermissive temperature is not an indirect effect of growth arrest, since AF8 cells kept quiescent for 48 h by serum starvation still have α-amanitin-sensitive activity (more than two-thirds of total nuclear activity; see Table I). Neither is it due to an aspecific effect of the high temperature since, under the same conditions, BHK cells (the parent cell line of AF8), maintain a high percentage of α-amanitin-sensitive RNA synthesis (Figure 8). Finally, it is not due to cell death, because, after reversal of the temperature, the cells are still capable of entering DNA synthesis (Figure 3) and nuclear

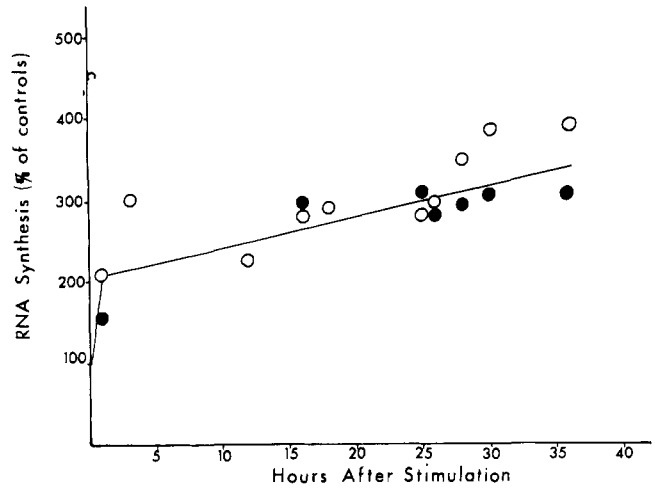

FIGURE 7: Nucleolar RNA synthesis in serum-stimulated confluent monolayers of tsAF8 cells. Resting cells were stimulated to proliferate as usual and harvested at the times indicated on the abscissa. Nucleoli were isolated and assayed for RNA synthesis as described in the text. (○—○) Cells stimulated at 32 °C; (●—●) cells stimulated at 40 °C. Each point is the average of at least two experiments.

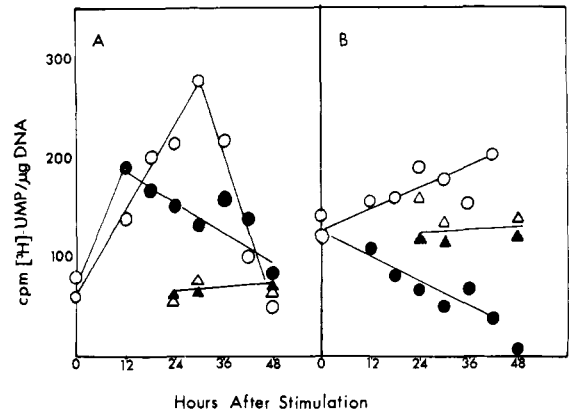

FIGURE 8: Effect of nonpermissive temperature on nuclear RNA synthesis in AF8 and BHK cells. Conditions similar to those described in Figure 5 and Table I. (A) α-Amanitin resistant RNA polymerase activity; (○—○) AF8 at 32 °C; (●—●) AF8 at 40 °C; (△—△) BHK at 32 °C; (▲—▲) BHK at 40 °C. (B) α-Amanitin sensitive RNA polymerase activity, same symbols as in A.

RNA synthesis is restored to high levels within 9–12 h (not shown).

The decrease in RNA polymerase II activity is reflected in the pool of the so-called "free" RNA polymerases, i.e., the RNA polymerase activity detectable by poly[d(AT)] in the presence of actinomycin D (Lampert & Feigelson, 1974). Table II shows that "free" RNA polymerase II activity increases in cells stimulated to proliferate at the permissive temperature, but not in cells stimulated at the nonpermissive temperature. In the latter, in fact, "free" polymerases II gradually disappear.

Discussion

In these experiments, RNA synthesis was determined by methods that measure, in essence, the endogenous RNA polymerase activity of isolated nuclei or nucleoli. There seems to be little doubt that the product of isolated nuclei or nucleoli is RNA (Reeder & Roeder, 1972; Marzluff et al., 1973; Grummt, 1975), but agreement on the meaning of the assay is not universal. However, it seems well established that, with both nuclei and nucleoli, their in vitro activity reflects their physiological activity in vivo (see review by Baserga, 1976, and also below). Keeping in mind the limitations of an in vitro system (Cox, 1976), our results show the following.

1. Serum stimulation at the permissive temperature (32 °C) causes in tsAF8 cells an increase in nuclear RNA synthesis which reaches its maximum at about the time when DNA synthesis reaches its peak.

2. At the nonpermissive temperature (40 °C) nuclear RNA synthesis increases as at 32 °C until the 16th hour after stimulation and then it decreases rapidly.

3. Nucleolar RNA synthesis increases markedly at both temperatures and remains elevated at 40 °C even after the 25th hour, declining only gradually. The effect of α-amanitin on nuclear RNA synthesis confirms that the decrease in nuclear RNA synthesis at the nonpermissive temperature is largely due to a decrease in RNA polymerase II activity.

It is well known that when resting cells are stimulated to proliferate RNA synthesis increases early in the prereplicative phase, that is, several hours before the onset of DNA synthesis (Lieberman et al., 1963). This increase in RNA synthesis has been reported in practically every population of cells stimulated to grow. It suffices to say here that it is detectable in whole cells (Lieberman et al., 1963), in isolated nuclei (Chiu & Baserga, 1975), in nuclear monolayers (Mauck & Green, 1973) and in isolated chromatin (Barker & Warren, 1966). While everybody agrees that rRNA synthesis is increased (Tsukada & Lieberman, 1964a,b; Nicolette & Babler, 1974; Schmid & Sekeris, 1975), there are differences of opinion on whether the increased synthesis is limited only to rRNA or it involves other RNA species. Some investigators (Mauck & Green, 1973; Rovera et al., 1974) believe that only synthesis of rRNA is increased, while transcription of other RNA species remains the same, although the amount of mRNA per cell may increase (Johnson et al., 1974). Using essentially the same methodologies (inhibition by α-amanitin), other investigators have reported an increased synthesis of both α-amanitin-resistant and α-amanitin-sensitive RNA species (Cooke & Brown, 1973; Organtini et al., 1975; Cox, 1976; Hardin et al., 1976). Our results are more in agreement with the latter investigators. However, the increase in α-amanitin-sensitive RNA synthesis is modest, ranging from 25 to 50% above the level of unstimulated controls and is not comparable to the fourfold increase in RNA polymerase II activity reported by Hardin et al. (1976) in the estrogen-stimulated uterus. More dramatic is the increase in α-amanitin-resistant RNA synthesis which reaches levels fourfold (or even higher) than those of controls. Under the conditions used in these experiments (1 μg/mL α-amanitin), α-amanitin-resistant RNA synthesis includes both RNA polymerase I and RNA polymerase III activities (Weinmann et al., 1976; Schwartz et al., 1974). However, the fourfold increase in nucleolar RNA synthesis indicates that an increase in the synthesis of preribosomal RNA is a prominent feature in the stimulation of total nuclear RNA synthesis. Several reports have indicated that pre-rRNA is the only recognizable RNA product that is synthesized by the nucleolus, either in vivo or in vitro (Yu & Feigelson, 1969; Grummt, 1975; Matsui et al., 1977). This has been shown by RNA-DNA hybridization, base composition and by homochromatography of the T_1 RNase digests (Ballal et al., 1977), and we had the opportunity of confirming it in our own laboratory (data not shown). In addition, the nucleolus apparently contain only RNA polymerase I (Coupar & Chesterton, 1975), which is specific for rRNA (Chambon, 1975). We can therefore conclude that stimulation of cell proliferation in AF8 cells causes a marked increase in rRNA synthesis, which can account for most, but not all, of the increase in total nuclear RNA synthesis. Nucleolar RNA synthesis increases also at the nonpermissive temperature (after serum stimulation) and remains elevated even after total nuclear RNA synthesis has declined. The small decrease observed could be easily explained by the fact that RNA polymerase I activity decreases in cells arrested in G_1 (see above).

At the nonpermissive temperature, RNA polymerase II activity is markedly affected. Indeed, at 40 °C RNA polymerase II activity decays steadily and, after 48 h, it is virtually absent. This result is in agreement with the in vivo experiments of Burstin et al. (1974) who showed that in tsAF8 cells at the nonpermissive temperature, [^{3}H]uridine incorporation into RNA decreases steadily for at least 30 h, after a brief increase in the first 5 h. After 48 h at the nonpermissive temperature, AF8 cells begin to die. The disappearance of RNA polymerase II activity from nuclei is not simply due to growth arrest, because serum-starved AF8 cells (arrested in G_0 for 48 h) still have a large amount of RNA polymerase II activity—two-thirds of the total activity and only slightly lower than in exponentially growing cells. It is also not due to an aspecific effect of the high temperature on RNA polymerase II, since the parent cell line of AF8 is not affected at 40 °C, neither in its growth properties nor in its RNA polymerase II activity. Neither is it due to an aspecific effect of the nonpermissive temperature on protein synthesis, since incorporation of radioactive amino acid into protein of AF8 cells at the nonpermissive temperature actually increases for the first 26 h and then levels off (Burstin et al., 1974). All this is not meant to suggest that RNA polymerase II itself is temperature sensitive in AF8. On the contrary the slow decay, longer than the reputed half-life of RNA polymerase II, given as 12 h (Goldberg & St. John, 1976), seems to indicate that the ts function is something else, perhaps a function that regulates the production of RNA polymerase II, or its presence and activity in nuclei. At any rate, the virtual absence of RNA polymerase II activity from AF8 nuclei could explain the failure of adenovirus 2 to replicate in AF8 cells at the nonpermissive temperature (Nishimoto et al., 1975), since adenovirus replication requires RNA polymerase II (Weinmann et al., 1974, 1976; Wilhelm et al., 1976). Furthermore, Nishimoto et al. (1977) by analysis of revertant clones showed that a single altered function restricts tsAF8 cell growth and prevents viral replication. It seems therefore that AF8 cells constitute an ideal system in which to ask how RNA polymerase II activity is regulated or what a decrease in RNA polymerase II activity

does to a cell. For instance, one could ask whether only nuclear RNA polymerase II activity is decreased or if indeed the amount of RNA polymerase II molecules in the cell has actually decreased. Or one could ask from these cells whether a decrease in RNA polymerase II activity will entail a decrease in the amount, diversity, and complexity of cytoplasmic mRNAs.

It is more difficult to relate the disappearance of RNA polymerase II activity to the block in the cell cycle. Shift-up experiments show that DNA synthesis is inhibited 60%, when cells are shifted up 12 h after stimulation, i.e., 8–18 h before the increase in DNA synthesis. From Table I it seems that an incubation of less than 20 h at 40 °C affects RNA polymerase II activity only modestly. The situation is further complicated by the fact that the shift-up and shift-down points do not coincide. Shift-down experiments constantly result in a delay of the entry into DNA synthesis proportional to the time spent at the nonpermissive temperature. The raises the old question of the existence of a "reset" mechanism in cells arrested in G_1 (Schneiderman et al., 1971) or in G_0 (Lindgren & Westermark, 1977).

Despite these reservations, some interesting conclusions can be drawn from these experiments, namely: (1) nucleolar RNA synthesis (and by extension, rRNA synthesis) is so increased in AF8 cells stimulated to proliferate, that it can account for most of the increase in total nuclear RNA synthesis; (2) the disappearance of RNA polymerase II activity at the nonpermissive temperature offers the possibility to study in AF8 cells the mechanism that regulate the activity of RNA polymerase II on one side, or, on the other side, the effect on the cell of its disappearance; and (3) in the almost virtual absence of RNA polymerase II activity, nucleolar RNA synthesis (i.e., RNA polymerase I activity) can still remain elevated.

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