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Reinitiation of synthesis of small cytoplasmic RNA species K and L in isolated HeLa cell nuclei in vitro

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ABSTRACT

Isolated HeLa cell nuclei were used to synthesize low molecular weight RNA species in-vitro. The labelled RNA released from the nuclei during the incubation mainly consists of 5S RNA, pre-tRNA and small cytoplasmic RNA species K and L. All these low molecular weight RNA species are synthesized by RNA polymerase C (or III). The polyanion heparin was applied to study the reinitiation of these RNA molecules in-vitro. A comparison of the kinetics of RNA synthesis in the absence and in the presence of this inhibitor demonstrates a highly efficient in-vitro reinitiation of scRNA species K and L as well as 5S and pre-tRNA by RNA polymerase C. These results indicate a general competence of this enzyme to catalyze the de-novo formation of specific gene products in-vitro.

INTRODUCTION

Due to the inherent problems in applying inhibitors of transcription such as α-amanitin to intact cells, isolated nuclei have been widely used to study eukaryotic RNA synthesis in-vitro (1-5). These cell-free systems provided evidence for the involvement of class A (or I), class B (or II) and class C (or III) RNA polymerases in the synthesis of ribosomal RNA, heterogenous nuclear RNA and 5S and tRNA, respectively (as reviewed in ref.6). In contrast to experiments studying the transcription of isolated DNA with purified RNA polymerases, the use of isolated nuclei unambiguously demonstrated the correct synthesis of specific gene products in-vitro, such as 5S and pre-transfer RNA (3-5,7), viral associated RNA (8,9) and correct RNA derived from structural viral genes (10). More recently, Weil et al. (11) described specific transcription of isolated

viral DNA with purified RNA polymerase II.

In addition to 5S and tRNA, eukaryotic cells contain a variety of other low molecular weight RNA species (12-16) which are located either in the cytoplasm (small cytoplasmic RNA,scRNA) or in the nucleus (small nuclear RNA,snRNA) (17). The small cytoplasmic RNA species K and L have been shown to be synthesized in isolated nuclei in-vitro. Results obtained by the use of α-amanitin demonstrated that RNA species K and L are synthesized by RNA polymerase C (18). In contrast, snRNA could not yet be obtained by in-vitro RNA synthesis. Indirect studies using inhibitors of transcription in-vivo, led to controversial conclusions as to which RNA polymerase is responsible for the synthesis of small nuclear RNA, either favoring polymerase A (18) or B (19). Yet another group of low molecular weight RNA species which are definitely synthesized by RNA polymerase I (snPI RNA) has been described in a variety of mammalian cells (20,21).

The RNA polymerase C catalyzed synthesis of 5S and pre-tRNA differs from other transcriptional systems by its ability to initiate the transcription of new RNA molecules with apparently high efficiency in-vitro. The present report studies the in-vitro synthesis of the low molecular weight RNA species K and L with respect to in-vitro reinitiation of these molecules in isolated HeLa cell nuclei.

MATERIALS AND METHODS

Isolation of nuclei and labelling of RNA in-vitro. HeLa S3 cells (kindly provided by Dr.S.Penman,MIT) were grown in suspension culture in Jokliks modified MEM medium (Gibco), supplemented with 5% calf serum. Prior to the isolation of nuclei, the cells were concentrated five fold and pretreated with 0.04 µg/ml actinomycin D for 45 minutes to inhibit nucleolar transcription. The isolation of nuclei was essentially as described by McReynolds and Penman (4). The washed nuclei from 2 x 10^7 cells were suspended in 150 µl suspension buffer (3.2mM $MgCl_2$; 10 mM KCl; 16mM Tris ph 8.0; 1.6 mM DTT; 80 mM $(NH_4)_2SO_4$ and 25% glycerol). Preincubation with 30 µl α-amanitin (10 µg/ml or 2 mg/ml) was for 10 minutes on ice. The reaction was started by the addition of start-mixture resulting in a final concentration of

0.1 mM ATP, GTP and CTP and 20 µCi of ^{3}H-UTP (41 Ci/mmole, Amersham, U.K.) per assay. No extra unlabelled UTP was added. The final volume of 280 µl was incubated at 25°C for the times indicated in the legends to the figures.

Extraction of RNA. The in-vitro RNA synthesis was terminated by chilling the nuclear suspension in an ice-bath. Subsequently the nuclei were pelleted by centrifugation at 2000 rpm for 3 minutes. The RNA released from the nuclei into the supernatant was phenol extracted as described previously (22) and collected by ethanol precipitation in the presence of 40 µg tRNA carrier. Heparin experiments were performed in the presence of 2 mg/ml heparin (Liquemin 5000,Roche) in the assay. In these experiments, RNA was extracted from the total assay mixture after digestion of the chromatin with 25 µg/ml DNAase I (Boehringer, Mannheim, Germany) in the presence of 0.5 M NaCl for 1 minute at 37°C. In order to remove the majority of the heparin, the RNA was centrifuged in 10% to 40% SDS-sucrose gradients in the SW 40 rotor of a Beckman centrifuge for 7 hours at 40,000 rpm. Fractions containing the labelled RNA were pooled, concentrated by ethanol precipitation and analyzed electrophoretically as described below.

Polyacrylamide gel electrophoresis. Acrylamide gel electrophoresis in 6% to 15% gradient slabgels with a 2.5% stacking gel was for 6 hours at 100 volts using the Laemmli buffer system(23). Fluorography of the gel with 2,5-diphenyloxazol - DMSO was as described in detail previously (17). Unless otherwise stated, exposure of the gel to the prefogged Kodak X Omat RP film was for eight days.

RESULTS

HeLa cell nuclei isolated by the use of the nonionic detergent NP 40 in the presence of hypotonic buffer are able to synthesize several low molecular weight RNA species in-vitro, which are released into the supernatant of the assay mixture. As shown in figure 1, in addition to 5S and pre-transfer RNA three distinct bands of RNA molecules can be resolved by electrophoresis in a 6% to 15% polyacrylamide slabgel. Two of these additio-

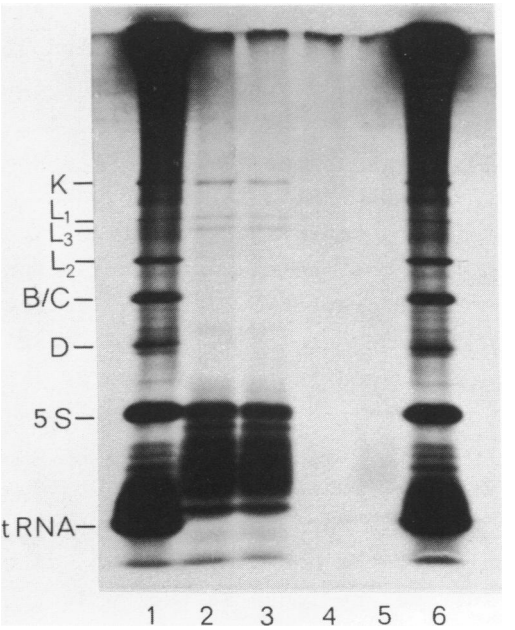

Figure 1. Gel electrophoresis of in-vitro synthesized low molecular weight RNA species.
RNA synthesis with nuclei isolated from cells pretreated with 0.04 µg/ml actinomycin D was for 20 minutes as described in methods. The RNA released from the nuclei was extracted and analyzed in a 6% to 15% polyacrylamide gel. Electrophoresis was at 100 volts for 6 hours. 1 + 6 = in-vivo labelled marker RNA; 2 = synthesis without α-amanitin; 3 = synthesis in the presence of 0.5 µg/ml α-amanitin; 4 = synthesis in the presence of 150 µg /ml α-amanitin; 5 = synthesis in the presence of 12 µg/ml actinomycin D.

nal bands, K and L_1, comigrate with the corresponding marker RNA, obtained by labelling cells in-vivo. The third band, designated as L_3, has no equivalent among the in-vivo RNA products. Under denaturating conditions such as 7 M urea, L_1 and L_3 form one single band (18), which comigrates with the single band formed by the in-vivo labelled scRNA conformers L_1 and L_2 under these conditions. Thus, the in-vitro synthesized L_3 apparently represents a third conformer of the scRNA species L. The in-vitro synthesis of RNA species K and L is resistant to 0.5 µg/ml α-amanitin but is completely sensitive to 150 µg/ml of this

inhibitor (figure 1, lane 3 and 4). This demonstrates that scRNA species K and L are synthesized by RNA polymerase C (or III). Furthermore, the labelling of all these small RNA molecules associated with the supernatant fraction is completely sensitive to 12 µg/ml actinomycin D (figure 1, lane 5). Therefore, these RNA products are synthesized in a template-dependent reaction.

The incorporation of radioactivity into RNA subsequently released from the nuclei reaches a plateau at about 20 minutes under our standard conditions of RNA synthesis, that is in the presence of 0.5 µg/ml α-amanitin (figure 2).
Further addition of nucleoside triphosphates at 20 minutes however, prolongs the synthesis of RNA for another 20 minutes with a linear rate of incorporation of nucleotides into RNA. RNA synthesis then starts to level off again. The readdition of nucleotides can be repeated several times resulting in a linear increase of RNA synthesis for at least ninety minutes. It should

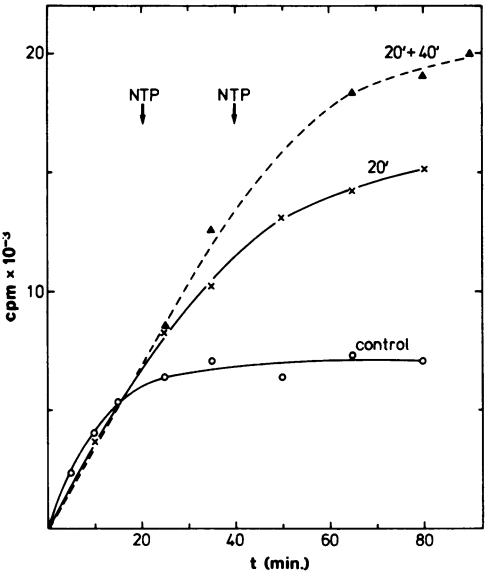

FIGURE 2. Kinetic analysis of in-vitro labelled RNA released from the nuclei.
In-vitro RNA synthesis was in the presence of 0.5 µg/ml α-amanitin with readdition of nucleotides at the points indicated by "NTP". (o——o) control; (x——x) nucleotides at 20 min.; (▲---▲) nucleotides at 20 and 40 minutes.

be mentioned that repeated addition of UTP alone, the concentration of which is two orders of magnitude below that of the other nucleotides in the assay, results in a similar resumption of incorporation of radioactivity into RNA.

The vast majority of labelled RNA which is released from the nuclei is 5S and pre-tRNA. Therefore, the reaction products of such a kinetic experiment described in figure 2, were analyzed by polyacrylamide gel electrophoresis. RNA synthesis was for up to eighty minutes, supplemented with additional nucleotides at 20, 40 and 60 minutes. Figure 3 shows a constant increase of radioactivity in low molecular weight RNA species K, L_1 and L_3 throughout the experiment and parallel to that of 5S and pre-tRNA.

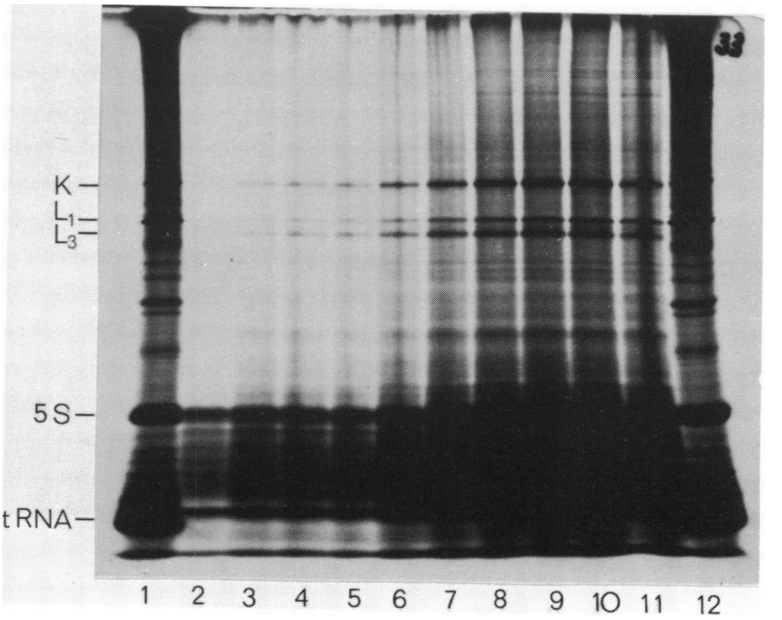

FIGURE 3. Gel electrophoresis of small RNA species synthesized for different times in vitro.
In-vitro RNA synthesis was as described in figure 2 with additional nucleotides added at 20, 40 and 60 minutes. 1 + 12 in-vivo labelled marker RNA; 2 to 11 corresponds to 5, 10, 15, 20, 30, 40, 50, 60, 70 and 80 minutes of RNA synthesis, respectively.

In the absence of reinitiation, incorporation of UMP into 5S and pre-tRNA ceases after 3 to 5 minutes (R. Reichel, unpublished) which means that one cycle of transcription is completed by that time. This indicates an average elongation rate of about 30 nucleotides per minute for the polymerase C catalyzed synthesis of these molecules. Since the size of RNA species K and L_1 with 260 and 220 nucleotides respectively, is about twice the chain length of 5S RNA one would expect the completion of these molecules within ten minutes at the latest. Therefore, the fact that radioactivity was incorporated into RNA species K and L for up to 80 minutes already suggests a reinitiation of these molecules in-vitro. In order to demonstrate this directly, in-vitro experiments were performed in the presence of heparin which inhibits initiation of RNA synthesis by procaryotic (24) as well as eucaryotic (25) RNA polymerases. In-vitro RNA synthesis was conducted as described with 2 mg/ml of heparin added after ten minutes of synthesis. It should be mentioned that in heparin experiments, RNA synthesis for 10 minutes without the inhibitor is necessary to get sufficient amounts of radioactivity incorporated for precise identification of individual bands in the autoradiograms. In the 5, 10 and 80 minutes control assays heparin was administered immediately after termination of RNA synthesis to ensure identical extraction conditions. The chromatin gel resulting from the heparin treatment was digested with DNAase I and the RNA was extracted as described. As heparin strongly interferes with gel electrophoresis of RNA molecules, the polyanion had to be removed prior to electrophoretical analysis. This was achieved by centrifugation of the RNA samples in SDS-sucrose gradients. Fractions corresponding to the 5S to 18S region of the gradients were pooled. This procedure excluded more than 90% of the heparin. The RNA was then analyzed in a 6% to 15% acrylamide gel as described in figure 3. When heparin was added at ten minutes of synthesis, no further increase of incorporation into RNA species K, L, 5S and pre-tRNA could be observed beyond twenty minutes (figure 4). The amount of label in all scRNA molecules remained constant during the following time points of the experiment. A comparison of the data of figures 3 and 4 indicates that frequent reinitiation takes place in the absence of heparin.

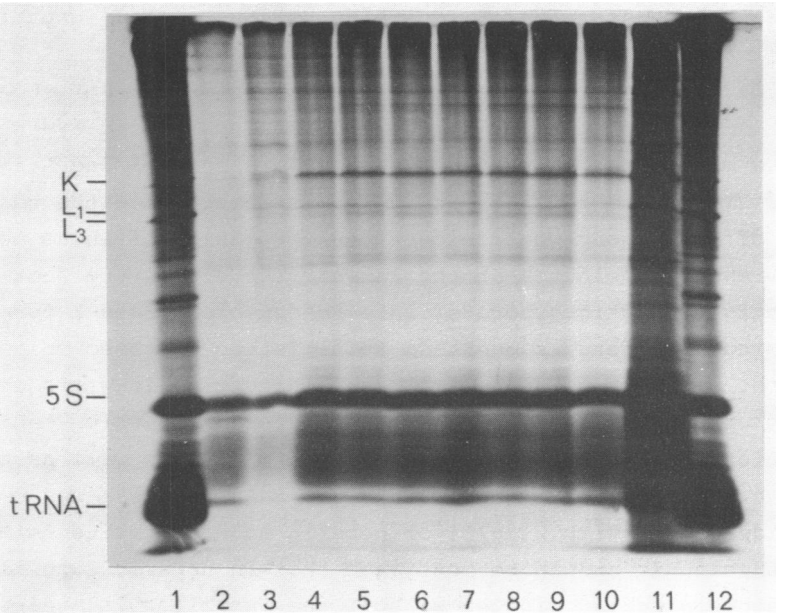

FIGURE 4. Gel electrophoresis of small RNA species synthesized for different times in-vitro in the absence and presence of heparin.

In-vitro RNA synthesis was as in figure 3 but with heparin added after 10 minutes. Heparin was removed prior to gel electrophoresis by centrifugation in SDS-sucrose gradients. 1 + 12 = in-vivo-labelled marker RNA; 2, 3 and 11 = synthesis without heparin for 5, 10 and 80 minutes, respectively; 4 to 10 corresponds to 20, 30, 40, 50, 60, 70 and 80 minutes of RNA synthesis with heparin added at 10 minutes, respectively.

Furthermore, the results of figure 4 reveal an identical response for the synthesis of low molecular weight RNA species K and L as well as 5S and pre-tRNA towards heparin and are in good agreement with results published by Udvardy and Seifart (5) with respect to the reinitiation of 5S and pre-tRNA synthesis in-vitro.

The slightly modified pattern of RNA products in figure 4 is due to the presence of heparin during the extraction of RNA, as can be seen from the 80 minutes control pattern (figure 4, lane 11). Addition of small amounts of heparin (0.2 mg/ml) alone to the labelled material released into the supernatant fraction results in a similar variation of the RNA pattern when analyzed

electrophoretically (not shown).

DISCUSSION

Isolated cell nuclei have been widely used as an intermediate transcription system between purified RNA polymerases on isolated DNA and intact cells. Two criteria are important in such in-vitro systems: The ability of the isolated nuclei (i) to catalyze the synthesis of specific RNA products in-vitro and (ii) to synthesize these products de-novo, including the initiation step. The polymerase C (or III) catalyzed synthesis of 5S and tRNA has been shown to unambigously with these requirements (3,5).

We were interested to study the in-vitro synthesis of low molecular weight RNA species other than 5S and tRNA by polymerase C and especially to see whether the enzyme also reinitiates the formation of scRNA species K and L in isolated nuclei. The polyanion heparin is known to completely suppress the initiation of transcription by RNA polymerases on purified DNA templates as well as within isolated nuclei. The comparison of the kinetics of formation of scRNA K and L in the absence and in the presence of heparin indicates that RNA polymerase C reinitiates very efficiently the in-vitro synthesis of these RNA molecules.

It appears that the ability to initiate the formation of new RNA chains in-vitro is a general characteristic of the enzyme molecule itself, rather than a special function of the genes which are transcribed by polymerase C. This assumption is supported by the finding that polymerase C also efficiently reinitiates the transcription of viral genes in-vitro (8). This ability further differentiates RNA polymerase C from the enzymes A and B and might hint to a different regulation of transcription of the latter enzymes. It cannot be excluded, however, that a reduced structural integrity of the isolated nuclei leads to a selective reduction of in-vitro reinitiation of high molecular weight RNA transcription. Nevertheless these data support the idea that isolated nuclei represent a powerful system to study individual steps of transcription in-vitro.

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