DNA POLYMERASE IN NUCLEOLI ISOLATED FROM EHRLICH ASCITES TUMOR CELLS

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SUMMARY: DNA replication was investigated in nucleoli isolated from Ehrlich ascites tumor cells. DNA synthesis was dependent on the presence of the four deoxynucleoside triphosphates and magnesium, but was reduced in the presence of ATP. The pH optimum for DNA replication was 8.5 to 9.0. N-Ethylmaleimide reduced the reaction significantly. DNA synthesis occurred on nucleolar chromatin and was stimulated by treatment of the nucleoli with a small amount of DNase I. Addition of exogenous DNA to the reaction mixture significantly stimulated [^{3}H]dTMP incorporation.

INTRODUCTION: Nucleoli are the major site of both biosynthesis of ribosomal precursor RNA and assembly of ribosomes(1). During the mitotic phase of the cell cycle, the nucleoli and nuclear membrane disappear and reorganized nucleoli appear in telophase (2,3). Therefore, in rapidly growing cells, such as Ehrlich ascites tumor cells, nucleoli must make large amounts of ribosomes and also replicate their own DNA to maintain nucleolar structures. Autoradiographic examination after incorporation of [^{3}H]thymidine into nucleolar chromatin in vivo (4,5) suggested that nucleolar DNA may be replicated without change in the normal structure of the nucleoli.

In the present studies, DNA replication was investigated in nucleoli isolated from Ehrlich ascites tumor cells. DNA synthesis in these nucleoli was dependent on the presence of the four deoxynucleoside triphosphates and Mg^{++} even without addition of exogenous DNA as template. Treatment of the

nucleolar chromatin with DNase I stimulated [^{3}H]dTMP incorporation into the nucleolar DNA.

MATERIALS AND METHODS:

Ehrlich ascites tumor cells(hyperdiploid strain) were inoculated into the peritoneal cavity of ddY mice and tumor cells were harvested 6 days later. Nucleoli were isolated as described previously(6). The isolated nucleoli, which were essentially free from nuclear contamination, were suspended in 0.34M sucrose containing 1 mM $MgCl_2$, dispersed by brief sonication and employed for DNA polymerase assay. Nucleoli were not disrupted under these conditions and still contained nucleolar chromatin. The DNA polymerase activity of the isolated nucleoli was assayed essentially according to the procedure used by Bernard and Brent(7), in studies on DNA replication of isolated HeLa cell nuclei, but ATP was omitted unless otherwise stated.

DNase activity was assayed as follows. [^{3}H]Thymidine-labeled nuclear DNA from Ehrlich ascites tumor cells was incubated with isolated nucleoli in buffers of various pH values, as described in the legend of Fig.2, in the presence of 5 mM $MgCl_2$, at 37°C for 60 min. After the reaction, the radioactivity recovered in the 0.5 N perchloric acid-soluble fraction was determined using toluene based scintillator fluid containing Triton X-100 and ethyl alcohol(1 : 0.25 : 0.25, by volume).

Pancreatic DNase I (RNase-free) was purchased from Worthington Biochemical Co., Freehold,N.J.. Pancreatic RNase A (type XI) and unlabeled deoxynucleoside triphosphates were obtained from Sigma Chemical Co., St.Louis,Mo.. [^{3}H]dTTP was from the Radiochemical Centre, Amersham.

RESULTS AND DISCUSSIONS:

Nucleoli were isolated from Ehrlich ascites tumor cells by sonication after stabilization with magnesium(6). Isolated nucleoli, usually 1 x 10^7 nucleoli per tube, were incubated as described in the legend to Fig.1. During incubation the incorporation of [^{3}H]dTMP into the acid insoluble material increased nearly linearly for 40 min without addition of exogenous DNA as template(Fig.1). DNA replication in the isolated HeLa cell nuclei was reported(7) to be completely dependent on the presence of ATP. However, the reaction in the isolated nucleoli was not dependent on ATP and was actually reduced in the presence of 5 mM ATP. As shown in Fig.1, in the absence of ATP the reaction rate only began to decrease

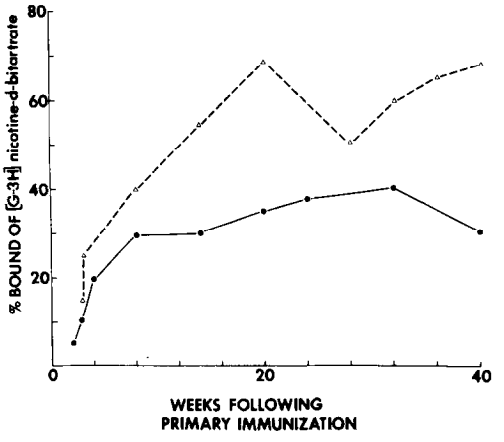

Fig.1. Time course of [^{3}H]dTTP incorporation into DNA of isolated nucleoli. The standard reaction mixture(150 µl) contained 8 mM $MgCl_2$, 0.5 mM each of dATP,dCTP and dGTP and 1.5 µM [^{3}H]dTTP(30 µCi/mmole) with 50 µl of buffer containing 120 mM Tris-HCl, pH 8.0, 22mM glucose, 0.1% Triton X-100, 1 mM EDTA, as described in ref.7., and 50 µl of 0.34 M sucrose containing 1 mM $MgCl_2$ and approx. $1x10^7$ nucleoli. The nucleolar suspension was sonicated briefly to disperse the nucleoli before incubation. The reaction mixture was incubated at 37° C and the reaction was stopped at the times indicated by addition of 5ml of ice cold 0.5N perchloric acid. The precipitate was washed 3 times with 0.5N perchloric acid and hydrolyzed by heating with 0.5ml of 0.5 N perchloric acid at 95°C for 45 min. Then radioactivity in the supernatant was determined in toluene based scintillator fluid containing Triton X-100 and ethyl alcohol (1 : 0.125 : 0.125, by volume). The DNA content of the supernatant obtained after hydrolysis was determined to correct for variation in the number of nucleoli per tube due to aggregation(13). When added, the final concentration of ATP was 5 mM.

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significantly after 40 min of incubation and so ATP was omitted from the reaction mixture in the present studies. The rate of [^{3}H]dTMP incorporation per 1 x 10^7 nucleoli was 0.10 pmole/30 min without ATP and 0.06 pmole/30 min with 5 mM ATP. This value for the rate of polymerization seems reasonable since the rate of [^{3}H]dTMP incorporation per 1 x 10^6 nuclei with ATP was 0.70 pmole/30 min under similar conditions. It was observed that [^{3}H]dTMP incorporation increased in proportion to the number of nucleoli, reaching saturation at above 4 to 5 x 10^7 nucleoli (data not shown).

Table I. Requirements for dTTP incorporation.

Reaction mixture	[^{3}H]dTMP incorporated (pmoles/mg DNA/30 min)		
Exp.I)			
Complete	9.1	(100%)	**
Minus dATP	5.9	(65)	
Minus dATP,dCTP	7.7	(85)	
Minus dATP,dCTP,dGTP	5.5	(60)	
Minus $MgCl_2$	3.2	(35)	
Plus ATP	3.8	(42)	
Exp.II)			
Complete	6.1	(100%)	**
Minus $MgCl_2$,plus $MnCl_2$	4.6	(70)	
Plus N-ethylmaleimide 2.5 mM	2.7	(44)	
10 mM	2.0*	(33)	
Plus native DNA (8 µg)	34.9*	(572)	
Plus heat-denatured DNA(8 µg)	23.4	(384)	

The complete reaction mixture in a final volume of 0.25 ml was as described in the legend of Fig.1. It was incubated for 30 min at 37°C. The rates of [^{3}H]dTMP incorporation into DNA with individual preparations of nucleoli were somewhat different. The numbers of nucleoli added were about 0.8×10^7 for Exp.I and 1.2×10^7 for Exp.II.
*; The sum of endogenous and exogenous DNA was used in calculating the specific activity. **; Percentage of value with the complete system.

The requirements for [^{3}H]dTMP incorporation are listed in Table 1. Magnesium appeared to be required for this reaction and its replication by manganese resulted in somewhat lower activity. Polymerization of dNTP was reduced on omission of one to three dNTP's from the reaction mixture. Due to the presence of endogenous dNTP's the requirement of four dNTP's for DNA replication in isolated nucleoli was not so clealy seen as with purified systems(8,9), but the general properties of DNA synthesis described in this paper suggest that terminal nucleotidyltransferase(10) did not contribute very much to the results obtained. N-Ethylmaleimide inhibited the DNA

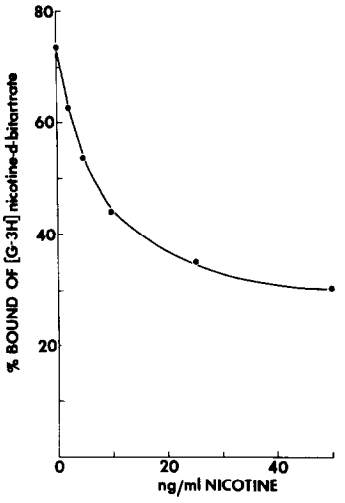

Fig.2. Effect of pH on the activity of DNA polymerase of isolated nucleoli. Polymerase was assayed in the standard reaction mixture described in the legend of Fig.1 (150 µl) with approx. $1x10^7$ nucleoli(50 µl), and the buffers(50 µl) at final concentrations of 40 mM: □ ,glycine; ▲ ,Tris-HCl; O ,phosphate; Δ ,acetate. The mixture for DNase assay contained ^{3}H-DNA from Ehrlich ascites tumor cells, nucleoli (approx. $1x10^7$), 5 mM $MgCl_2$ and the same buffers as for polymerase assay. The total count of ^{3}H-DNA per tube was $1x10^4$ cpm. Therefore, less than 1 per cent of the substrate added was released into the acid-soluble fraction by endogenous DNase in the nucleoli.

polymerase in the nucleoli significantly,but about 30 per cent of the activity was still observed in the presence of 10 mM N-ethylmaleimide. Addition of either native or heat denatured DNA from Ehrlich tumor cell nuclei significantly stimulated the DNA polymerase activity.

The pH optimum for [^{3}H]dTMP incorporation into the endogenous nucleolar chromatin was 8.5 to 9.0(Fig.2). The DNase activity in the nucleoli was also maximal in this pH range (Fig.2). Thus it may be concluded from this experiment that lack of DNA polymerase activity in the neutral pH range was not due to the presence of a DNase with a pH optimum at about neutrality. The alkaline pH optimum of the DNA polymerase of the isolated nucleoli is compatible with that of

Table II. Effects of nucleases on [^{3}H]dTTP incorporation.

Addition	Time of addition after start of incubation(min)	Total incubation time(min)	[^{3}H]dTMP incorporated (pmoles/mg DNA)
None	-	20	5.4
None	-	60	8.0
RNase(4μg/tube)	0	20	3.0
RNase(4μg/tube)	20	60	6.6
DNase(4μg/tube)	0	20	14.1
DNase(4μg/tube)	20	60	19.0

The incubation mixture consisted of a final volume of 0.25ml of the standard reaction mixture described in the legend to Fig.1. Where specified, 4 μg of RNase A or DNase I were added. Before use RNase A was heated at 80°C for 10 min in acetate buffer (pH 5.0) to reduce the DNase activity. Approx. 1.2×10^7 nucleoli were used per tube.

the low-molecular-weight DNA polymerase purified from nuclei (11), while the sensitivity to N-ethylmaleimide is similar to that of cytoplasmic, high-molecular-weight DNA polymerase(11). These facts suggest that complex reactions were involved in DNA synthesis in the isolated nucleoli.

Preliminary experiments were made on the effects of DNase and RNase. As shown in Table 2, addition of RNase either with the enzyme (nucleoli) or 20 min later, partially reduced incorporation of dTTP. On the other hand, under similar conditions,DNase stimulated the incorporation of dTTP. This increase in incorporation was due to a gap-filling reaction(12) on the nucleolar chromatin, that is a reaction with DNase I activated chromatin, and the amount of DNase I added(4μg/tube) was not enough to cause release of digestion products from the chromatin. These results indicated the occurrence of at least two reactions, one which was RNase sensitive and another which was concerned with gap filling.

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