^{3}H-dTTP INCORPORATING ACTIVITIES IN ISOLATED RAT LIVER NUCLEI*

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SUMMARY Based on differential inhibitions produced by deletion of substrates and ATP and by addition of sulfhydryl-blocking agents and actinomycin D, three distinct processes incorporate ^{3}H-dTTP into DNA of nuclei isolated from rat hepatocytes. One activity occurs only in nuclei isolated from S-phase cells and appears to be the DNA replicating process. The other two activities occur in both proliferating and non-proliferating nuclei and represent terminal transferase and, possibly, repair polymerase; these processes are activated by damaging nuclei either during or after isolation.

Several recent studies have described synthesis of DNA by isolated nuclei which provide both polymerase and primer-template (1-10). Conditions *in vitro* which promote incorporation of labeled deoxyribonucleoside triphosphates into DNA have varied widely, suggesting that different enzymatic processes were involved (10). This paper reports that at least three activities that incorporate ^{3}H-dTTP into DNA of isolated rat liver nuclei can be distinguished by their differential responses to variations in reaction mixtures.

Materials and Methods Nuclei were obtained from intact livers (hepatocytes not proliferating) or from residual livers 20 hr following 70% partial hepatic resection (hepatocytes rapidly proliferating) of 200-250 gm male Wistar albino rats (11). Nuclei were isolated by one of two methods: either with low shearing

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forces in a dilute homogenate (7) or with high shearing forces in a concentrated homogenate (12). Incorporation of ^{3}H-dTTP into DNA of nuclei took place in a basal assay mixture of the composition listed in Table 1. Alterations of the basal mixture are noted in the tables. Reactions were routinely terminated after 15 min at 37°C; DNA was separated and quantitated and radioactivity assayed as previously described (10).

Results and Discussion Consistent with the differing rates of DNA synthesis in regenerating and normal livers in vivo (7, 11) regenerating nuclei isolated by a low-shear, dilute homogenate method (7) are over 20 times as active in vitro as are nuclei from normal liver (Table 1). Incorporation of ^{3}H-dTTP is separable into ATP-dependent and ATP-independent fractions. Sulfhydryl blocking agents markedly inhibit ATP-dependent activity but they are ineffective against ATP-independent incorporation. ATP-dependent activity in nuclei from regenerating liver is almost completely blocked by high concentrations of actinomycin D (act D), whereas ATP-dependent activity in nuclei from normal liver is insensitive to this compound. ATP-independent activity is inhibited by 60 to 75% by act D. Deletion of unlabeled deoxyribonucleoside triphosphates (dNTPs) affects the two ATP-dependent activities (act D-sensitive and act D-insensitive) and the ATP-independent activity differently. ATP-dependent, act D-sensitive activity is almost totally suppressed by deletion of one or more unlabeled dNTPs, whereas ATP-dependent, act D-insensitive activity is augmented by unlabeled dNTP-deletions. ATP-independent activity is partially inhibited by deletion of unlabeled dNTPs (50 to 70% when all three are absent). These data demonstrate three activities that incorporate ^{3}H-dTTP into DNA of isolated rat liver nuclei. Type A is dependent on ATP, sulfhydryl groups,

Table 1

INCORPORATION OF ^{3}H-dTTP INTO DNA OF LIVER NUCLEI ISOLATED WITH LOW SHEAR IN A DILUTE HOMOGENATE

(DPM/μg DNA)

Reaction Mixture	Nuclei from Normal Liver			Nuclei from Regenerating Liver		
	with ATP	without ATP	ATP-dependent	with ATP	without ATP	ATP-dependent
Basal[a]	36[b]	30	6[c]	812	101	711
-dCTP	45	25	20	79	65	14
-dCTP,dGTP	53	21	31	60	50	10
-dATP,dCTP,dGTP	58	18	40	58	49	9
+act D[d]	21	12	9	47	36	11
+act D,-dNTPs	43	12	31	29	21	8
+NEM[e]	37	39	0	68	80	0
+p-HMB[f]	47	40	7	68	107	0

[a] Basal mixtures (150 μl) contained nuclei (20-40 μg DNA); 15 μmole Tris-HCl, pH 7.4; 2.4 μmole KCL; 0.6 μmole $MgCl_2$; 1.2 μmole 2-mercaptoethanol; 0.3 μmole ATP; 3.4 mμmole each of dATP, dCTP, and dGTP; and 0.67 mμmole ^{3}H-dTTP (0.05 Ci/μmole).

[b] Basal incorporations are means of up to 30 replicates run on separate days; deletion and addition data are normalized to basal values.

[c] ATP-dependent incorporation is the difference between incorporations in the presence and absence of ATP.

[d] Nuclei were exposed to 1 to 1.5 μg actinomycin D/μg DNA for 30 min at 4°C before and during incubation.

[e] Nuclei were exposed to 5×10^{-4} M n-ethylmaleimide in buffered sucrose for 30 min at 4°C, before incubation.

[f] p-Hydroxymercuribenzoate (1.5×10^{-4} M) was present in mixtures during incubation.

template and presence of all four deoxyribonucleoside triphosphates. Type B is dependent on ATP and sulfhydryl groups, but independent of template and is inhibited by presence of more than one deoxyribonucleoside triphosphate. Type C is independent of ATP or sulfhydryl groups, but partially dependent on template and presence of all four deoxyribonucleoside triphosphates.

Activities of types B and C can be augmented by treating isolated nuclei with small amounts of pancreatic deoxyribonuclease (DNase I) or by isolating these organelles with a high-shear, concentrated homogenate method (12). As compared to untreated liver nuclei from normal rats, incorporation of ^{3}H-dTTP into DNA is augmented about 10-fold by DNase treatment (Table 1); both types B and C are increased but type A activity is not induced. As compared to nuclei isolated by a low-shear, dilute homogenate method (7), total activity in normal nuclei isolated by a high-shear, concentrated homogenate method (12) is increased, while that in similarly isolated regenerating nuclei is decreased (Table 3). In both normal and regenerating nuclei, activities of types B and C are increased, but type A activity in regenerating nuclei is decreased. Because of these changes, total incorporation of ^{3}H-dTTP by DNA of normal and regenerating nuclei prepared by this method differs by a much smaller extent (2 to 2.5-fold) than do rates of DNA synthesis *in vivo* (10 to 100-fold) (7,11). If type A activity is to be evaluated, nuclei should be isolated by a method producing low shear in a dilute homogenate.

Autoradiographic studies by us and by Lynch *et al* (7) show that type A incorporation occurs only in nuclei isolated from cells that are in S-phase *in vivo*. Thus, type A activity appears to represent continuation *in vitro* of DNA synthesis begun *in vivo* (replicative polymerase); incorporation of ^{3}H-dTTP *in vitro* is

Table 2

EFFECT OF TREATMENT OF ISOLATED NORMAL NUCLEI WITH DNase ON INCORPORATION OF ^{3}H-dTTP INTO NUCLEAR DNA

Reaction Mixture	(DPM/μg DNA) with ATP	without ATP	ATP-dependent
Basal	343	196	147
-dCTP	376	190	186
-dCTP,dGTP	405	170	235
-dATP,dCTP,dGTP	600	102	498
+act D	258	91	167
+act D,-dNTPs	625	95	530
+NEM	214	200	14
+p-HMB	170	186	0

Nuclei were exposed to 0.003 to 0.005 μg pancreatic deoxyribonuclease/μg DNA for 5 min at 37°C and then washed before incubation.

See footnotes in Table 1.

semiconservative (7). We have been unable to induce type A activity *in vitro*. Activities of types B and C can occur in all nuclei, regardless of their position in the cell cycle *in vivo* (10); incorporation of ^{3}H-dTTP *in vitro* is not conservative. Types B and C activities can be initiated *in vitro* by various manipulations that affect the conformation and integrity of DNA (for example, creation of single-strand breaks and 3'-hydroxyl termini with DNase I). Type B activity clearly represents terminal transferase (13, 14); type C is identical to that previously studied by us and hypothesized to represent repair polymerase (10).

The analogy between DNA polymerizing activities in isolated hepatocyte nuclei and those in bacteria made artificially perme-

Table 3

INCORPORATION OF ^{3}H-dTTP INTO DNA OF LIVER NUCLEI ISOLATED WITH HIGH SHEAR IN A CONCENTRATED HOMOGENATE

(DPM/μg DNA)

Reaction Mixture	Nuclei from Normal Liver			Nuclei from Regenerating Liver		
	with ATP	without ATP	ATP-dependent	with ATP	without ATP	ATP-dependent
Basal	150	108	42	393	148	245
-dCTP	150	92	58	157	83	74
-dCTP,dGTP	170	78	92	141	78	63
-dATP,dCTP,dGTP	180	51	129	110	69	41
+act D	60	18	42	91	46	45
+act D,-dNTPs	212	-	-	156	-	-
+NEM	110	101	9	124	130	0
+p-HMB	100	99	1	109	112	0

See footnotes in Table 1.

able (15) appears close. Type A nuclear activity and bacterial polymerase II (putative replicative polymerase) share common characteristics, as do type C and bacterial polymerase I (putative repair polymerase). Characteristics of nuclear activities of types A and C are also similar to those of two DNA polymerases recently separated from nuclei of HeLa cells (16). An enzyme with major properties similar to those of type A activity has also been partially purified from rat liver (17). It is noteworthy that requirements for ATP by nuclear activities of types A and B are stringent, whereas neither presumptive replicative polymerase (17) nor terminal transferase (13, 14) requires this nucleotide for *in vitro* activity after these enzymes are "purified." Further study of DNA polymerizing processes in isolated nuclei and nuclear fractions may clarify this dichotomy, as well as provide insight into the mechanics and regulation of DNA replication and repair in eukaryotic cells.

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