DEVELOPMENT OF TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE ACTIVITY IN EMBRYONIC CALF THYMUS GLAND*

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SUMMARY

Analysis of extracts of calf embryo tissues for deoxynucleotide polymerizing enzymes showed that terminal deoxynucleotidyl transferase is present only in thymus and the levels of activity increase with growth of the fetus.

INTRODUCTION

Terminal deoxynucleotidyl transferase, isolated from calf thymus gland, is an enzyme with no known biological function. Its demonstrated activity is to catalyze the addition of deoxynucleotidyl residues from deoxynucleoside triphosphates to the 3'-hydroxyl group of preformed polynucleotide chains. No template requirement has been demonstrated for this enzyme, in contrast to DNA polymerase isolated from the same source[1]. Biochemical interest in the terminal transferase stems partly from its possible relation to DNA polymerase, and partly from its practical uses in the synthesis of single chain polydeoxynucleotides and oligodeoxynucleotides [2,3]. This communication demonstrates that terminal transferase arises during the early embryonic stages in the calf and appears to be unique to thymus. These findings enhance interest in establishing the biological role for terminal transferase, and may provide a new parameter for studying thymus biology.

EXPERIMENTAL PROCEDURES

The original work on terminal deoxynucleotidyl transferase[4] demonstrated

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that deoxynucleotide initiators as short as trinucleotides or as long as linear ØX-174 DNA were active provided a free 3'-OH group was present. The usual assay system for this enzyme contains a trinucleotide initiator and a single deoxynucleoside triphosphate. This method does not permit detection of the activity in crude extracts of thymus, although it is useful with purified fractions[1]. An attempt to solve this problem in continuing fractionation work[5] used two approaches: analytical fractionation on sucrose gradients, and the use of short polydeoxynucleotide initiators (chain length about 300) and dGTP as substrate. The rationale is that fractionation on gradients may remove inhibitory activities, as well as endogenous acceptors. The use of polydeoxynucleotide initiators attenuates endonuclease effects, since limited chain breaks in polydeoxynucleotide will not reduce the amount of acid-insoluble product scored. The use of dGTP monomer produces a chain addition that self associates[6], thereby reducing the effectiveness of exonuclease contamination. The soluble terminal transferase has been purified to homogeneity[5] and its behavior on sucrose gradients is known. The use of gradient fractionation adds molecular weight constraint (transferase = 32,460; polymerase $\geq$ 100,000) as well as reaction type to the analytical procedure. These modifications in assay procedure permit reasonable estimates of the total terminal transferase in crude extracts of calf thymus, and probably other tissues as well.

Tissues are homogenized in a glass tube homogenizer with a teflon pestle using five volumes of 40 mM potassium phosphate (pH 7.4), 40 mM NaCl in 1 mM mercaptoethanol as the homogenization medium. The homogenates are clarified in the Sorvall centrifuge at 8000 g and then centrifuged in the Spinco L at 105K g. The 105K g supernatant fraction is precipitated at 70% saturation with $(NH_4)_2SO_4$ and the protein pellet obtained by centrifugation is dissolved in 0.1 M potassium phosphate (pH 7.5). The concentrated fraction is then dialyzed overnight against the same buffer. The concentrated, dialyzed soluble extract (0.25 ml) is loaded onto a 5-20% (w/w in 0.1 M potassium phosphate, pH 7.5) sucrose gradient and centrifuged for 16 hours at 40,000 rpm in the SW50 rotor

of the Spinco L-2 centrifuge. Gradients are fractionated by displacement from the bottom of the tube with 40% sucrose to produce 24 to 30 equal volume fractions. Fractions are assayed using filter disks[7] for terminal transferase and DNA polymerase activities as previously described[5], and in some cases exonuclease activity with ^{14}C-polydeoxyadenylate as substrate. The nuclear extracts are prepared by extraction of the 8000 g pellets with 1 M NaCl in 0.1 M potassium phosphate[9], centrifugation at 105K g, and dialysis overnight against 0.1 M potassium phosphate (pH 7.5). Concentration and analysis of nuclear extracts are the same as for the soluble extracts. Extracts concentrated by pressure dialysis give equivalent results.

RESULTS

The initial assumption in surveying a number of tissues was that termi-

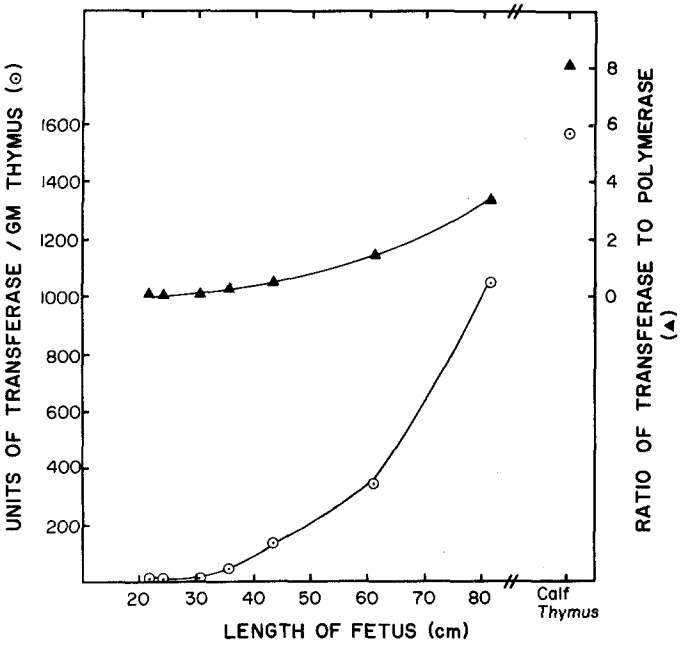

Figure 1--Development of terminal deoxynucleotidyl transferase in embryonic calf thymus gland. Soluble extracts of fetal calf thymus glands were analyzed for terminal transferase activity and DNA polymerase activity on sucrose gradients as described in the text. ^{3}H-dGTP with specific activity of 10^4 cpm per nmole was used for transferase assay, and ^{14}C-dTTP with specific activity of 5-7 x 10^3 cpm per nmole was used for DNA polymerase assay. Units of enzyme activity per gm of thymus were calculated by summing the activity on the gradient. One unit of terminal transferase is defined as one nmole of ^{3}H-dGMP incorporated per hour. One unit of DNA polymerase is defined as one nmole of ^{14}C-dTMP incorporated per hour.

nal transferase activity might be related to DNA polymerase as an active subunit or degradation product. Analysis for terminal transferase and DNA polymerase activities in a variety of calf fetal tissues on gradients demonstrated that only thymus gave terminal transferase activity at the sensitivity used. The analytical procedure easily detects 4 units per gram of tissue, one unit being one nmole of nucleotide polymerized in one hour. A series of fetuses was then examined for transferase and the result shown in Figure 1 (also Table 1) was obtained. This set of gradient analyses (typical results in Figure 2) clearly shows that enzyme activity develops during the early embryonic stages of the calf and persists into early adolescence. The amount of terminal transferase per gram of thymus tissue increases by two orders of magnitude during thymic development, while the DNA polymerase activity remains sensibly constant (Table 1).

A number of calf fetus tissues have been analyzed for terminal transferase; including spleen, lung, liver, muscle, brain and kidney. All are

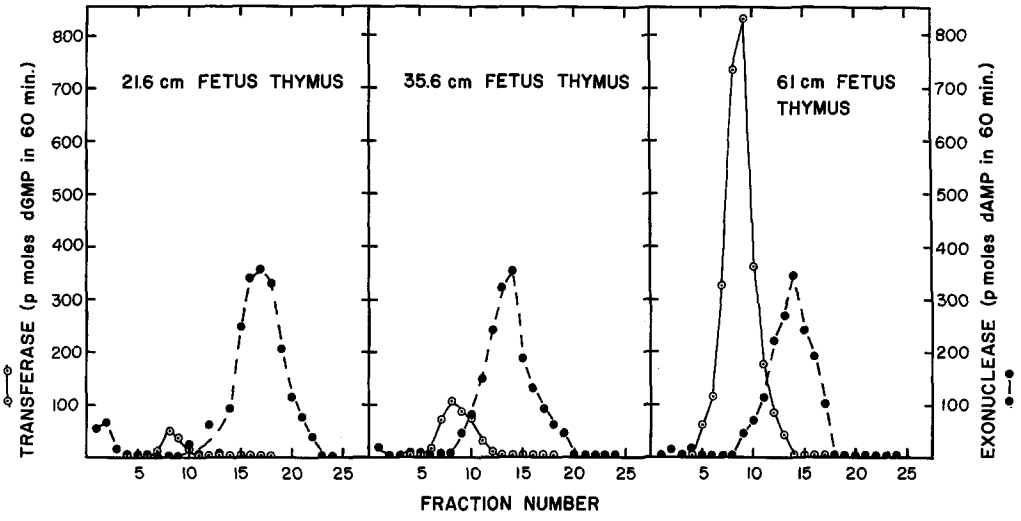

Figure 2--Sucrose gradient analysis of fetal calf thymus at various stages of development. Soluble extracts were prepared and analyzed on sucrose gradients as described in text. Exonuclease activity was determined on 10 µl of each gradient fraction by incubation with 50 µl of a mixture of 0.1 M in potassium cacodylate (pH 7.5), 4 mM $MgCl_2$, and 20 µM ^{14}C-polydeoxyadenylate (chain length about 100 and specific activity of 2800 cpm per nmole) for 1 hour at 35°. The decrease in acid-insoluble radioactivity was measured by liquid scintillation counting.

TABLE 1

Survey for DNA Polymerase and Terminal Deoxynucleotidyl Transferase

		TDT	DP
Bovine:			
22 cm fetus thymus	soluble*	+(17)	+(207)
24 cm fetus thymus	soluble	+(8)	+(236)
31 cm fetus thymus	soluble	+(18)	+(219)
36 cm fetus thymus	soluble*	+(49)	+(193)
44 cm fetus thymus	soluble	+(140)	+(344)
61 cm fetus thymus	soluble	+(342)	+(240)
81 cm fetus thymus	soluble*	+(1049)	+(309)
calf thymus	soluble	+(1570)	+(194)
	nuclear	+(710)	+(36)
16 cm fetus lung	soluble	-	+
16 cm fetus liver	soluble	-	+
16 cm fetus muscle	soluble	-	+
16 cm fetus brain	soluble	-	+
43 cm fetus kidney	soluble	-	+
43 cm fetus liver	soluble	-	+
43 cm fetus spleen	soluble	-	+
43 cm fetus lung	soluble	-	+
47 cm fetus spleen	soluble	-	+
buffy coat	soluble	-	+
cervical lymph node	soluble	-	+
Porcine:			
thymus	soluble	+	+
mesenteric lymph node	soluble	-	+
spleen	soluble	-	+

		TDT	DP
Rat:			
thymus	soluble*	+	+
liver	soluble	-	±
spleen	soluble	-	+
	nuclear	-	±
intestinal mucosa	soluble	-	+
Peyer patches	soluble	-	+
	nuclear	-	±
bone marrow	soluble	-	+
Chicken*:**			
thymus	soluble	+	+
bursa Fabricus	soluble	-	+
	nuclear	-	+
Human:			
bone marrow	soluble	-	±
spleen	soluble	-	+
Rabbit*:**			
thymus	soluble	+	+
	nuclear	+	+
bone marrow	soluble	-	+
	nuclear	-	+
spleen	soluble	-	+
	nuclear	-	+
Tissue culture cells:			
mouse L-cell	soluble**	-	+
Novikoff hepatoma	soluble**	-	+

Number in parentheses are units per gram of tissue.
*Nuclear extracts also positive, but not tested on gradients.
**Nuclear extracts also negative, but not tested on gradients.
***Frozen tissues obtained from Pel-Freez Biologicals. All other tissues collected fresh.

negative as tested here. Table 1 also lists results from tissues of other species. Since the thymus is a lymphoid organ it was of interest to see if terminal transferase might occur in other lymphoid tissues. While the majority of the tissues in Table 1 contain easily detectable levels of DNA polymerase, only thymus shows activity in the transferase assay. Rabbit lymphoid tissues under antigenic stimulus are also devoid of transferase.

Although the analytical procedure used in the work presented here is fairly rigorous, it is possible that all negative results have some trivial explanation. One way to test for false negatives is to carry out mixing experiments with active and inactive extracts. Not all negative results in Table 1 have been tested in this manner, but Figure 3 shows gradient patterns of mixed extracts of calf fetus spleen and fetus thymus, and no indication of inhibition is found. Lymph node extracts and fetus thymus extracts with low activity have also been tested against active calf thymus extracts and in no case has inhibition been found. These results indicate the absence of inhibitor in the in-

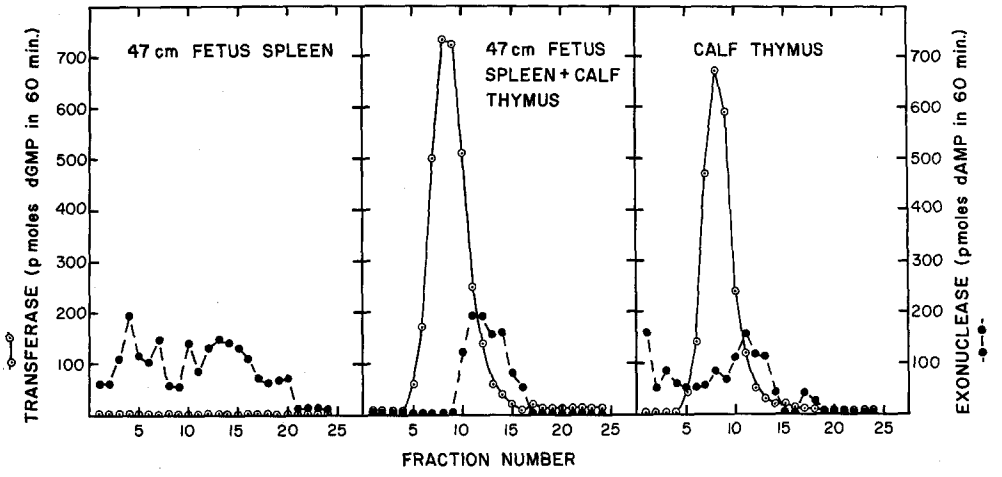

Figure 3--Effect of fetal spleen extract on terminal transferase activity in calf thymus extract. Extracts of 47 cm fetus spleen and calf thymus were prepared as described in the text. Frame 1 is a mixture of 0.1 ml of concentrated spleen extract and 0.1 ml of 0.1 M potassium phosphate (pH 7.5), frame 2 is 0.1 ml of concentrated spleen extract plus 0.1 ml of concentrated thymus extract, and frame 3 is 0.1 ml of concentrated thymus extract plus 0.1 ml of 0.1 M potassium phosphate (pH 7.5). All were analyzed on sucrose gradients for terminal transferase activity and exonuclease activity.

active extracts. It is also important to note that exonuclease activity is present in the gradients, but because of the terminal transferase assay used little effect on transferase activity is seen.

DISCUSSION

The data presented allow two conclusions to be drawn. First, terminal transferase is localized in thymus, appearing in the thymus of all species so far examined, and is not found in other tissues (Table 1). Second, terminal transferase appears during the process of embryonic development and seems to persist in the thymus of adolescent and young animals.

In searching for possible biological function for terminal transferase, it may be useful to recall that no nucleolytic or pyrophosphorylytic activities have been demonstrated with the purified enzyme [5,10]. For this reason it is not possible to assign a degradative role to the enzyme. The relationship between terminal transferase and DNA polymerase also needs examination since the transferase may be a degradation product or active subunit of polymerase. In this regard, the original set of experiments was designed to examine a variety of tissues for these activities with fetal tissues providing a source of samples that proceed generally from a high rate of DNA synthesis (and DNA polymerase) to a reduced rate as differentiation continues. If terminal transferase were a normal cellular degradation product of DNA polymerase it would seem that all such tissues would produce detectable levels of transferase. This does not seem to be the case. The possibility that there is some special degradation mechanism for DNA polymerase leading to terminal transferase in thymus remains as a possible explanation for the increasing level during fetal development.

The most intriguing suggestion is that the terminal transferase is related to some special function of thymus cells, particularly their role in immune reactions. This possibility is amenable to experiment and should be resolved directly. While the detailed resolution of function for terminal transferase remains an unsolved problem, it is of considerable interest that the

enzyme is specific to thymus and has a developmental cycle. At the present time that remains as the primary conclusion of this investigation.

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