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CLONING AND EXPRESSION OF A cDNA ENCODING A CATALYTICALLY ACTIVE FRAGMENT OF CALF THYMUS DNA POLYMERASE ALPHA

Kimberly Foster[1], Kersten Lüthi-Steinmann[2], Marjorie Barnes[1], Gary McMaster[3], Elena Ferrari[2], Knut Eliassen[1,4], Naseema Khan[1], Neal Brown[1*], and Ulrich Hübscher[2]

[1] Department of Pharmacology, University of Massachusetts Medical School, Worcester, MA 01605

[2] Department of Pharmacology and Biochemistry, University of Zürich-Irchel, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

[3] Max-Planck Institute for Immunobiology, Postfach 1169, D-7800 Freiburg-Zähringen, West Germany

[4] Permanent address: Department of Physiology, Norwegian College of Veterinary Medicine, Postboks 8146, Oslo Dep., Norway

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A calf thymus cDNA expression library was constructed in the EcoRI site of λgt11 and probed with an antibody raised against calf thymus DNA polymerase α. Three classes of antibody-reactive clones were isolated. The largest class carried a 1.9 kilobase calf cDNA insert and expressed a 165-175 kilodalton ß-galactosidase:calf fusion protein which displayed DNA polymerase activity. The characteristic responses of the polymerase activity to alpha-specific inhibitors and antibodies identified the 1.9 kilobase cDNA as a sequence specifically derived from the structural gene encoding the pol α catalytic core.

The mutual interest of our laboratories in the structure and function of mammalian DNA pol α (1) has led us to collaborate in an effort to clone and to engineer for expression a complete cDNA sequence encoding the enzyme's catalytic core. The first stage of our effort, which we report here, has been to clone a partial, "probe" sequence with which to select the

*Author to whom correspondence should be addressed.

Abbreviations: pol α, DNA polymerase alpha; ß-gal, E.coli ß-galactosidase; BuAdATP, N^2-butylanilino-2'-deoxyadenosine-5'triphosphate; IgG, immunoglobulin type G; MOAB, monoclonal antibody; POAB, polyclonal antibody; pol I, DNA polymerase I; Kd, kilodaltons; Kb, kilobases; pfu, plaque-forming units; ELISA, enzyme linked immunospecific assay; IPTG, isopropyl-ß-D-thio-galactopyranoside; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; dNTPs, 2'deoxyribonucleoside 5'-triphosphates.

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appropriate full length transcript and its structural gene. The screening strategy for probe isolation has been implemented in two steps. The first screen has exploited a calf thymus cDNA:λgt11 expression library (2) and an antibody raised against calf pol α to select "candidate" clones expressing ß gal:calf fusion proteins. The second screen, designed to identify a candidate cDNA as pol α-specific, has exploited DNA polymerase activity assay of candidate fusion proteins to detect catalytic activity characteristic of calf DNA pol α.

MATERIALS AND METHODS

Cloning Reagents and Inhibitors. λgt11 and E.coli strains Y1088 and Y1089 were propagated as described (2). Restriction enzymes and EcoRI linkers were from P.H. Stehelin (Basel). BuAdATP (3) and aphidicolin (4) were from, respectively, Drs. G. Wright and S. Spadari.

Antibodies were IgGs purified on protein A-Sepharose (Pharmacia), using manufacturer's instructions; the purified IgGs were further adsorbed with E.coli 1089 protein-agarose (2) before use. MOAB 132-20 (5) was derived from ATCC mouse hybridoma line CRL1640; mouse IgG P3 (5) was a gift of Dr. T. Wang. Goat anti-ß gal (POAB-ß gal, ref 6) was a gift from Dr. Ry Young. Neutralizing antibody specific for E.coli DNA pol I (POAB-pol I) was from Dr. L. Loeb. POAB-pol α, the polyclonal, pol α-specific probe reagent, was raised in a New Zealand White rabbit, using the method of Barnes and Brown (7) and an antigen consisting of an holoenzyme form of pol α purified approximately 7000-fold from calf thymus (Fract. VI, ref. 8). Immunoblot and activity gel analysis, the results of which are not shown, indicated that the POAB-pol α: (a) strongly bound and neutralized the polymerase activity of the major 63 Kd, catalytic core peptide of the fraction VI calf pol α, and (b) did not bind or inhibit the activity of calf thymus DNA polymerases ß and γ or terminal deoxyribonucleotidyl transferase.

Construction and Screening of cDNA Library. RNA was extracted from surgically-excised thymus from a week-old calf by the method of Nielsen et al (9) and used to prepare poly A^+ RNA (10). The poly A^+ mRNA was reverse transcribed and EcoRI-linkered by the method of Huynh et al (2), and the linkered DNA was size selected (2) for fragments >0.2 Kb and ligated into the EcoRI site of λgt11 (2). The DNA was packaged into phage and the product used to infect E.coli Y1088, yielding approx. 10^6 pfu/μg and an insert frequency >0.9. Screening exploited the methods of Huynh et al. (2) and employed 10^5 pfu per 150 mM petri dish, POAB-pol α as the primary probe antibody, and an ELISA detection system based on the use of peroxidose-linked goat anti-rabbit IgG (Miles, ref. 11). Seven signal-positive clones were isolated; their DNAs were extracted and sorted by restriction and Southern hybridization analysis (12); three classes were identified. Class III consisted of 5 clones, and classes I and II consisted of one clone each. Classes I, II, and III contained EcoRI-resolvable calf cDNA inserts of, respectively, 0.5, 1.0, and 1.9 Kb. The class III insert strongly hybridized with the class II insert but did not hybridize with the class I insert, even in conditions of low stingency.

Preparation, Induction and Extraction of λgt11 Lysogens. E.coli Y1089 was lysogenized, induced with IPTG, and harvested as described (2); cell pellets were extracted by freeze-thawing (2) in selected buffers; for SDS-PAGE, Laemmli buffer (13) was used. AG-buffer (14) was used for activity gel analysis, and for immunoselection of fusion protein, the following buffer was used: 100 mM Tris:HCl (pH 7.5), 10 mM EDTA, 1 mM phenylmethysulfonyl fluoride, 1 mM pepstatin, and 10 mM sodium metabisulfite. Extracts contained 5-15 mg protein per ml.

Immunoselection. Analytical, protein A-based immunoselection employed POAB-ß gal, formalinized S.aureus A (Pansorb, BioRad.), and the method supplied with the bacterial reagent. Preparative immunoselection exploited Protosorb Lac Z Immunoaffinity Adsorbent (Promega Biotech), an agarose matrix covalently bound to a mouse MOAB specific for E.coli ß-gal. The matrix-bound fusion protein was prepared, adsorbed and eluted with 0.1 M $NaHCO_3/NA_2CO_3$ (pH 10.8) as described by the manufacturer; samples were neutralized immediately upon collection with excess Tris:HCl (pH 8.0).
Miscellaneous Methods. SDS-PAGE employed the method of Laemmli (13), activity gel analysis, the method of Spanos and Hübscher (14), and conventional DNA polymerase assay, the method of Ottiger and Hübscher (15). Assay of antibody-specific neutralization of DNA polymerase activity employed the method of Tanaka et al (5).

RESULTS

Characteristics of the ß Gal:calf Fusion Proteins. The left panel of Fig. 1 displays the results of SDS-PAGE of extracts of IPTG-induced wild type λgt11 and the recombinant lysogens. The λgt11 lysogen (lane 3), as expected, yielded a 116 Kd ß-gal band. In each of the recombinant lysogen extracts, the ß-gal band was absent and replaced by a novel protein species; the unique proteins of recombinant classes I, II and III migrated at, respectively, 130 Kd (lane 4), 150 Kd (lane 5) and 165-175 (lane 6). Immunoblot analysis (method of ref. 16; results not shown) of the extracts of Fig. 1 with POAB ß-gal and POAB-pol α identified the novel bands in lanes 4-6 as the expected ß-gal:calf fusion proteins. The results of size analysis of the three classes of cDNA and their IPTG-inducible proteins were consistent with the production, in each recombinant lysogen, of the expected

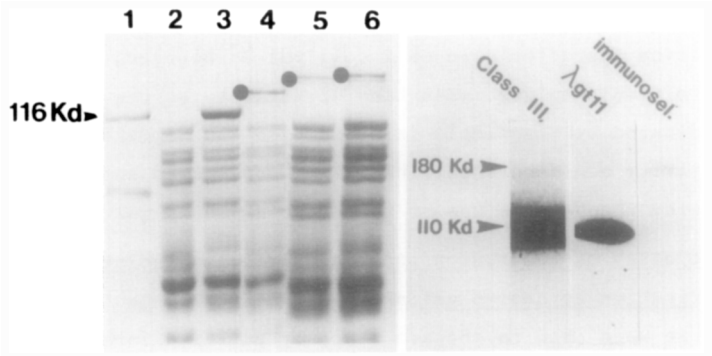

Fig. 1. Analysis of ß gal:calf fusion proteins.
Left panel, SDS-PAGE of extracts of recombinant lysogens in E.coli Y1089. Each lysogen was induced with IPTG and extracted as described in Methods; except for lane 1, 300 µg of extract protein was used in each gel slab. Lane 1, purified E.coli ß-gal control; lane 2, E.coli Y1089; lane 3, λgt11 lysogen; lanes 4, 5, and 6, respective extracts of lysogens I, II, and III. Dots indicate positions of the putative fusion proteins.
Right panel, DNA polymerase activity gel analysis of the lysogens of λgt11 and the class III recombinant. Y1089 lysogens were induced with IPTG, extracted, and 300 µg of extract protein were analysed (see Methods), using 3 hours' autoradiographic exposure. Center lane, λgt11 extract; left lane, class III extract; right lane, the product of protein immunoselection (Pansorb, see Methods) of approximately 300 µg of the class III lysogen extract.

114 Kd truncated ß-gal (2) linked in tandem to a complete or near complete translation product of the respective calf cDNA insert.

The Class III Fusion Protein Displays Polymerase Activity Characteristic of Calf Pol α.

Detection of activity in crude extracts. Our effort to detect an active fusion protein exploited the Y1089 lysogen of the largest, class III recombinant. The first step employed direct assay of crude extracts of an IPTG-induced λgt11 (control) lysogen and the comparable class III lysogen, and it exploited aphidicolin (4) and BuAdATP (3) to distinguish pol α-specific activity from the heavy E.coli DNA polymerase background. We found that 4-5% of the activity of the class III crude extract was susceptible to the latter pol α-specific inhibitors; the corresponding extract of wild type λgt11 lysogen did not contain such an activity.

Activity gel analysis. Encouraged by the results with crude extracts, we sought to link the pol α-like activity of the class III lysogen to its ß-gal:calf fusion protein. Our initial approach exploited the technique of denaturing activity gel analysis (14); the latter method provided an ultrasensitive assay (17) and facilitated direct evaluation of the activity and molecular weight of the protein in conditions which minimized proteolysis, a problem which often besets ß-gal fusion proteins in the λgt11 system (2). The results of one of our experiments are shown in the right panel of Fig. 1; the λgt11 and class III lanes compare the respective activity profiles of extracts of the induced λgt11 lysogen with that of the class III lysogen. Both contained a major activity band at the 110 Kd position characteristic of E.coli pol I; however, the class III extract displayed a unique, weak band of activity at the position of 165-175 Kd. As indicated by the single faint band of the "immunosel." lane of Fig. 1., the activity could be specifically immunoselected and separated from the pol I activity through the use of the Staph A/POAB-ß-gal method.

Direct assay of the fusion protein-polymerase isolated by preparative immunopurification on anti-ß gal:agarose. Using the Lac Z Immunoaffinity Adsorbent, we were able to prepare a highly purified form of the class III protein with sufficient activity to be susceptible to conventional assay and analysis. We made the following observations regarding the properties of the fusion protein and its immunopurification. (1) The DNA polymerase activity of the purified protein represented approximately 1-2% of the total polymerase activity of the crude, class III lysogen extract; (2) no comparable immunoselectable activity could be detected in IPTG-induced extracts of the wild type λgt11 lysogen; (3) like the activity immunoselected via the protein A method (cf. Fig. 1 Immunosel. lane), the product migrated in activity gel analysis as a band at 170-175 Kd, and (4)

TABLE I· Properties of the Immunopurified Class III Fusion Protein, Calf Pol α, and E.coli Pol I

	ACTIVITY (%)[1]		
CONDITION	(A) FUSION PROTEIN	(B) POL α	(C) Pol I
Complete	100	100	100
-DNA	<2	<2	-
$-Mg^{+2}$	<5	<5	-
-dATP, dCTP, dGTP	9	11	-
+Aphidicolin (50μg/ml)[2]	<2	<2	102
+BuAdATP (2.5μM)[3]	<1	<1	105
+0.3μg POAB-pol α IgG (Preimmune)	110	102	100
+0.3μg POAB-pol α IgG (Immune)	59	16	100
+0.2μg MOAB-132-20 IgG (Immune)	52	7	-
+0.2μg Mouse P3 IgG (Control)	102	105	100
+1μg POAB-pol I	100	100	<5

[1]Assayed by the method of ref. 15; units per assay:(A), 0.5; (B), 1; (C), 1.2.
[2]Ki of aphidicolin for calf pol α, <0.5μg/ml; for fusion protein, <1μg/ml.
[3] Ki of BuAdATP for calf pol α, <0.2μM; for fusion protein, <0.2μM.

the collected properties of the polymerase activity of the immunopurified protein, summarized by Table I, below, were characteristic of calf pol α.

The immunopurified activity was dependent on primer:template DNA, divalent cation, and dNTPs. The activity was conventionally susceptible to aphidicolin and BuAdATP, and was completely resistant to POAB-pol I (E.coli). The activity was susceptible both to the POAB-pol α used to select the class III clone and to the MOAB 132-20, which reacts with the conserved active-site epitope of several pol alphas (5). However, the fusion protein activity was not as sensitive as natural pol α to the latter antibodies. We do not yet know the basis for the latter resistance, although we believe it likely that critical antibody-reactive pol α epitope(s) of the class III protein may be masked or, in part, obliterated via its fusion to the truncated ß gal.

DISCUSSION

The POAB used to probe the λgt11 expression library was raised against an holoenzyme form of pol α - a form which contained not only the catalytic core of the enzyme, but several other proteins. Considering the antigen's heterogeneity, we recognized that the cDNAs of antibody-reactive clones might generate proteins which were irrelevant to the pol α core. (The class I cDNA, which does not hybridize with the sequences common to the class II and III inserts, may be such a non-core sequence). Therefore, we were compelled to provide proof that a candidate cDNA was, indeed, a bona fide core-specific sequence; to obtain such proof we sought to demonstrate the presence of pol α-like catalytic activity in the largest, class III fusion protein.

Our rationale for expecting that the λgt11 expression system would yield a catalytically active fragment of pol α was fueled by two observations. First, Hübscher et al (18) and Hässig (8) had found that the catalytic activity of calf pol α is retained in core fragments as small as 60 Kd. The second observation, derived from recent comparisons (19) of the primary sequences of pol α-like viral DNA polymerases, is that the active site region is likely encoded in the 3' third of the relevant polymerase-specific transcript. Considering the latter observations, we hypothesized that expression of pol α-specific cDNAs enriched for 3' sequences (i.e. oligo(dT)-primed reverse transcription of poly A+ mRNA) should have a reasonably high probability of yielding a catalytically competent protein, particularly if the protein had a mass in the range of 50-60 Kd and were protected at its NH_2 terminal end by fusion to a "neutral" peptide sequence such as the truncated ß-gal peptide of the λgt11 system.

In sum, the results we have obtained indicate that the clone class III calf cDNA encodes the functional active site region of bovine DNA pol α. Considering the weight of the evidence, we cannot readily construct an alternative, trivial explanation. The activity behaved as a typical λgt11-specific ß-gal fusion protein; it was absent from the λgt11 parental lysogen; its detection in the recombinant depended on IPTG induction; its gel mobility was consistent with that predicted on the basis of the size of the calf cDNA insert, and the activity was specifically selectable with antibodies specific for ß-gal.

The sensitivity of the polymerase activity of the recombinant-specific protein to pol α-specific antibodies and to aphidicolin and BuAdATP indicates that the catalytic activity was simply not that of another polymerase which might share a POAB-reactive, active site epitope. If the activity were that of calf pol ß or pol γ, it would be expected to display resistance to both inhibitors (3). If the activity were that of the pol α-like calf DNA pol δ, it would be expected to be sensitive to aphidicolin and resistant to BuAdATP (20).

Future Directions. Our present line of investigation with the class III sequence, like that taken by Johnson et al. (21) with the cloned sequences of the pol I gene of yeast, is aimed at: (1) the dissection of the structure of the bovine pol α gene(s); (2) the isolation and the sequence characterization of maximum length calf pol α-specific transcripts, and (3) the expression and characterization of *in vitro* translation products of pol α-specific mRNAs, and engineered cDNA versions thereof. We hope that the class III calf cDNA sequence will be generally useful to investigators of mammalian pol α as a sequence probe with which

to isolate and manipulate pol α-specific sequences of mammalian cells other than those of Bos taurus.

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