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The evolutionary conservation of DNA polymerase α

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ABSTRACT

The evolutionary conservation of DNA polymerase α was assessed by immunological and molecular genetic approaches. Four anti-human KB cell DNA polymerase α monoclonal antibodies were tested for their ability to recognize a phylogenetically broad array of eukaryotic DNA polymerases. While the single non-neutralizing antibody used in this study recognizes higher mammalian (human, simian, canine, and bovine) polymerases only, three neutralizing antibodies exhibit greater, but variable, extents of cross-reactivity among vertebrate species. The most highly cross-reactive antibody recognizes a unique epitope on a 165-180 kDa catalytic polypeptide in cell lysates from several eukaryotic sources, as distant from man as the amphibians. Genomic Southern hybridization studies with the cDNA of the human DNA polymerase α catalytic polypeptide identify the existence of many consensus DNA sequences within the DNA polymerase genes of vertebrate, invertebrate, plant and unicellular organisms. These findings illustrate the differential evolutionary conservation of four unique epitopes on DNA polymerase α among vertebrates and the conservation of specific genetic sequences, presumably reflective of critical functional domains, in the DNA polymerase genes from a broad diversity of living forms.

INTRODUCTION

DNA polymerase α is a key component of the cellular chromosomal replication apparatus and is generally accepted as the principal polymerase in eukaryotic DNA replication. Several investigators have described the purification of DNA polymerase α from a variety of eukaryotic sources and have observed a remarkably similar set of constituent polypeptides. Specifically, most reports have demonstrated a group of large molecular weight polypeptides, ca. 125-180 kDa (1-10), which contain the catalytic activity of polymerase α; two lower molecular weight polypeptides, ca. 55 and 46 kDa (1-10), which contain DNA primase activity (1,3,11); and the presence of an intermediate molecular weight polypeptide, ca. 70 kDa (1-3,6-8,10), which exhibits no known enzymological activity and has been shown in various systems to be a tightly associated component of the DNA

polymerase/DNA primase complex (6,7,10,12). The similar polypeptide composition of this essential and ubiquitous enzyme in such phylogenetically diverse organisms suggests the possibility of strong evolutionary pressures to conserve critical functional domains on the DNA polymerase α molecule.

We have utilized both immunologic and molecular genetic approaches to investigate this possibility. By employing four of a panel of sixteen anti-human DNA polymerase α monoclonal antibodies (13) in a series of immunoassays with polymerase α isolated from cell lines of representative phylogenetic classes, we demonstrate the differential evolutionary conservation of four unique epitopes on the catalytic polypeptide of DNA polymerase α. Further analysis with different segments of the cDNA (14) of the human DNA polymerase α catalytic polypeptide in genomic Southern hybridization studies with DNA from many representative species, indicates the presence of several highly conserved DNA sequences among organisms as phylogenetically disparate as primates and unicellular fungi.

MATERIALS AND METHODS

Cell lines used in the immunoassays, human KB cells, simian CV-1, rodent LA-9, Pekin Duck, Gekko Lung GL-1, Xenopus laevis and Baboon lymphoblast were purchased from ATCC. Chinook Salmon Embryo (CHSE) cells were a gift from Dr. Joanne Leong, Oregon State University. Calf thymus DNA polymerase α was a gift from Dr. L.M.S. Chang, Uniformed Services University of the Health Sciences. DNA samples used in genomic Southern hybridization were from human KB cells, a human cell line containing 4 X chromosomes (GM1202A) from the NIGMS Human Cell Repository, calf thymus DNA from Calbiochem and rodent cell DNA from the LA-9 cell line. Xenopus DNA from the Xenopus laevis kidney cell line (XTC) was a gift from Dr. J. Yun Tso, Stanford University. Drosophila DNA and yeast DNA were from W. Seagrave and W. Weiss of Stanford University, respectively. DNA samples from tobacco and green algae (CC125) were gifts from Dr. Madeline Wu of the University of Maryland.

Monoclonal anti-KB cell DNA polymerase α antibodies and nonimmune control P3 IgG, were prepared and purified as described (13). Covalently linked IgG-Sepharose 4B was prepared as in (10). The growth and extraction of various cell lines were carried out as described in the ATCC manual or as reported (6,10,15). The preparation and assay of DNA polymerase α fraction IIA, the definition of the polymerase α unit, and the binding and neutralizing assays of antibodies with polymerase α fractions, using nonimmune P3 IgG as control, have been described (6,10,13).

Immunoprecipitation of polymerase α antigens from crude extracts of in vivo labeled cells was performed as described (10) with one modification; the IgG-Sepharose beads were preincubated with RIPA buffer (16) containing 1% gelatin at 4°C for 30 min. to reduce non-specific protein-Sepharose IgG interactions.

Genomic Southern hybridization was performed with 5 μg each of EcoR1 digested samples of human, calf, rodent, tobacco, green algae and yeast DNA, with Hind III digested Xenopus DNA and with Pst1 digested Drosophila DNA. The digested genomic DNA samples from different species were run on a 0.8% agarose gel in a Tris.acetate buffer system and transferred onto Gene Screen Plus membrane (17). The membrane was prehybridized in 20% formamide, 3XSSC, 5X Denhardt solution, 1 mM NaPyrophosphate, 1% SDS, 100 μg of heat denatured E.coli tRNA, and 1 μg each of EcoR1 digested and heat denatured pBR322 and pcD L-1 DNA (to eliminate vector cross hybridization) at room temperature for at least 16 hours. The membrane was then hybridized in 20% formamide, 3XSSC, 5X Denhardt solution, 5% dextran sulfate, 1% SDS, 1 mM of NaPyrophosphate, 100 μg of heat denatured E. coli tRNA and 3 to $6X10^6$cpm/ml of labeled cDNA fragment (14). The cDNA probes used and the labeling of these probes were as described (14,18). Hybridization was performed at 42°. C with rotation for at least 16 hours. The blot was first washed two times, 15 minutes each, with 2X SSC and 0.1% SDS at room temperature followed by three 45-minute washes with the same buffer at the designated temperature. The blots were radioautographed on Kodak XAR-5 film at -70° C with a Dupont Lightning Plus intensifier screen. Each Southern blot was exposed for an appropriate length of time to achieve a signal intensity comparable to that of the human 4 X chromosome DNA lane.

RESULTS

Demonstration of four unique and differentially conserved DNA polymerase α epitopes

The phylogenetic survey was performed with polymerases derived from both transformed and non-transformed cell lines. Previous investigations (M. A. Miller, unpublished observation) indicated that the four monoclonal antibodies used in this study do not detectably distinguish among DNA polymerases α from transformed and non-transformed cells under the conditions of these assays. However, the purity of the enzyme fraction does affect the extent of reactivity with these antibodies. The study was thus performed by incubating a constant quantity of DNA polymerase α activity

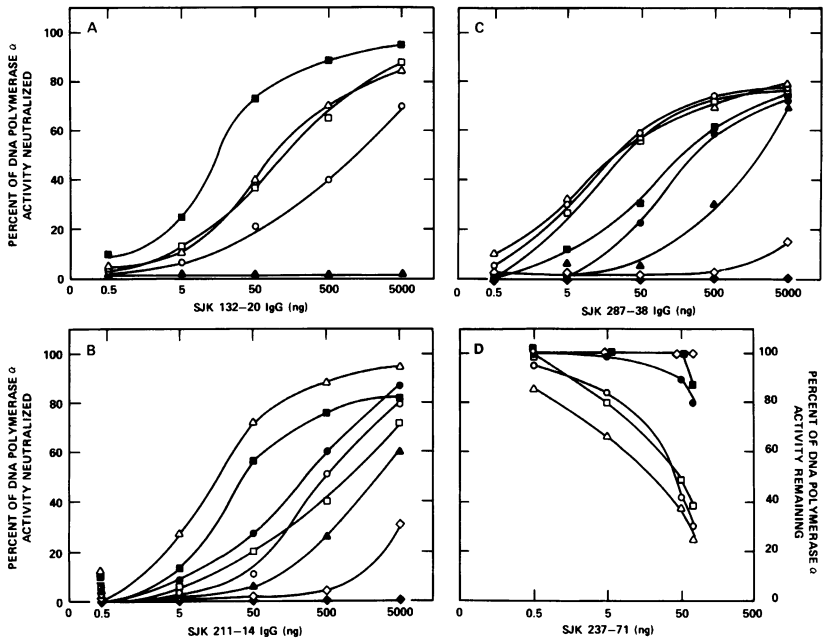
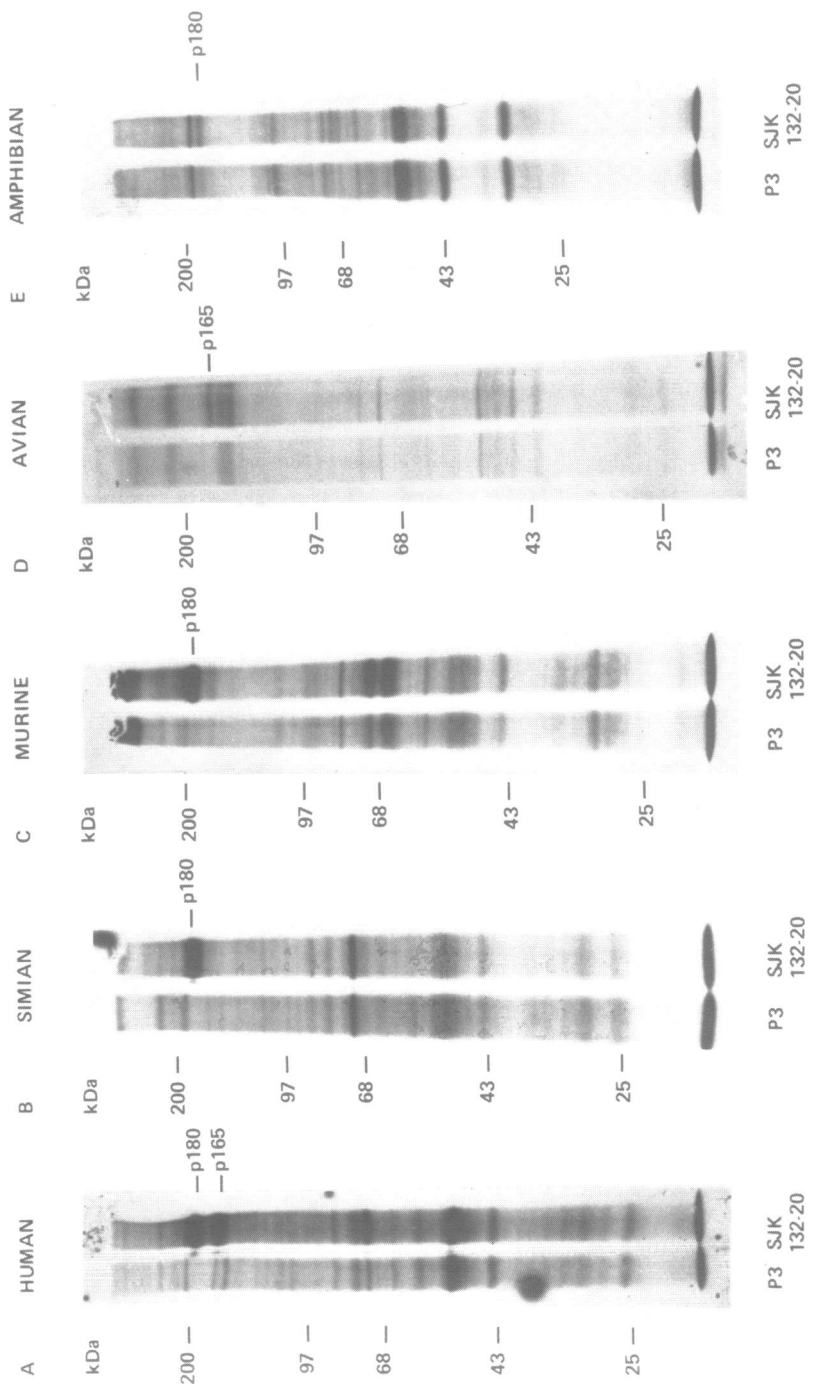


Figure 1. Reactivities of monoclonal anti-human DNA polymerase α antibodies with eukaryotic DNA polymerase α from various phylogenetic divisions. 0.5 unit of DNA polymerase α (fraction IIA) from human (KB), simian (Baboon lymphoblast and monkey CV-1), canine (dog thymus), bovine (calf thymus), murine (LA-9), avian (Pekin duck), reptilian (Gekko), amphibian (Xenopus laevis), or piscine (CHSE) cell extracts was incubated with neutralizing antibody SJK 132-20, SJK 211-14, or SJK 287-38 and surviving polymerase α activity was measured as described (13,15). For the non-neutralizing antibody assay, 0.5 unit of DNA polymerase α (fraction IIA) from each cell line was incubated with non-neutralizing antibody SJK 237-71, and polymerase activity in the supernatant was measured as described (13). (A) Reactivity of antibody SJK 132-20 with vertebrate polymerase α activities. Percent of mammalian (human, simian, canine, bovine and murine) (■), avian (△), reptilian (○), amphibian (□), or piscine (▲) cell polymerase α activity neutralized. (B) Reactivity of neutralizing antibody SJK 211-14 with vertebrate polymerases. Percent of human (△), canine (■), bovine (●), baboon (□), monkey (○), murine (▲), or avian (◇) cell polymerase α activity neutralized; (◆), percent of either reptilian, amphibian, or piscine cell polymerase α activity neutralized. (C) Reactivity of neutralizing antibody SJK 287-38 with vertebrate polymerases. Symbols for each species are the same as in (B). Values of 2.22% ± 5.8% (mean ± 2SD) polymerase activity neutralized are defined as background. (D) Reactivity of non-neutralizing antibody SJK 237-71 with a panel of eukaryotic DNA polymerases α. Percent of human (△), baboon(□), monkey (○), bovine (●), canine (■) cell polymerase α activity remaining; (◇), percent of all other lower vertebrate (murine, avian, reptilian, amphibian, and piscine) cell polymerase α activity remaining. Values of 98.2% ± 8.0% (mean ± 2SD) polymerase activity remaining in supernatant are defined as background.

(0.5 unit) of comparable purity (fraction IIA) from each cell line with increasing amounts of antibody under both neutralizing (for antibodies SJK132-20, 211-14, 287-38) and binding (SJK 237-71) conditions (13). The results, Fig.1, show that each antibody reacts with the panel of vertebrate polymerases α in a unique and independent way, a result that corroborates our earlier finding that each of these antibodies recognizes a unique epitope on the human DNA polymerase α molecule (10). The most cross-reactive of the antibodies, SJK132-20, exhibits virtually identical reactivity with all of the mammalian polymerases α and retains a strong ability to neutralize polymerase activity from lower vertebrate cell extracts as distant as the amphibian, Fig.1A. However, this antibody does not recognize epitopes on fish cell polymerase α, Fig.1A, or on DNA polymerse α from Drosophila and yeast (M. A. Miller, unpublished observation and (13)). The other two neutralizing antibodies, SJK211-14 and 287-38, are able to neutralize all mammalian polymerases α tested, and are both weakly cross-reactive with avian polymerases, but fail to neutralize polymerase activity from reptilian, amphibian, and fish cell extracts, Fig.1B and C. There are at least two distinct explanations that can account for the loss of antibody reactivity with the lower vertebrate polymerases; one, that the epitopes are no longer present on these polymerases; or two, that the epitopes are present but altered such that antibody binding does not neutralize polymerase activity. To distinguish between these two formal possibilities, each neutralizing antibody was tested in a binding assay with those polymerases which it failed to neutralize and was found to be unable to recognize the polymerases tested (data not shown). We conclude, therefore, that these specific epitopes are no longer present on DNA polymerase α in the lower vertebrates.

A similar series of experiments shows that the non-neutralizing antibody SJK237-71 exhibits strong cross-reactivity only with higher mammalian (simian) polymerases. There is weak cross-reactivity with calf and dog thymus polymerases and lack of cross-reactivity with any of the more phylogenetically remote polymerases from rodent to fish, Fig 1D. Since this antibody does not neutralize activity, it is reasonable to conclude that the SJK237-71 epitope is in a non-catalytic domain that is not essential for polymerase activity. Thus, one would expect that there had been less evolutionary pressure for the maintenance of this site on the polymerase molecule.

While these data generally reflect the known phylogenetic relationships amongst the vertebrate species, three anomalous results are noted that are at


A
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25 —
p180
p165
P3 SJK 132-20

variance with this scheme. First, the SJK132-20 antibody appears to neutralize amphibian (Xenopus) polymerase in preference to the reptilian (Gekko) polymerase, Fig 1A; second, the SJK211-14 antibody neutralizes simian polymerase to a much lesser extent than both human and other mammalian (bovine and canine) polymerases, Fig 1B; and third, all three neutralizing antibodies neutralize the canine polymerase in preference to the bovine enzyme, while the binding antibody, SJK237-71, cross-reacts with these two enzymes in an opposite manner. Despite these relatively minor incongruities, for which we have no explanation, the data taken together demonstrate a strong correlation between the strength of the antibody-polymerase interaction and the evolutionary divergence of the polymerase antigen epitope.

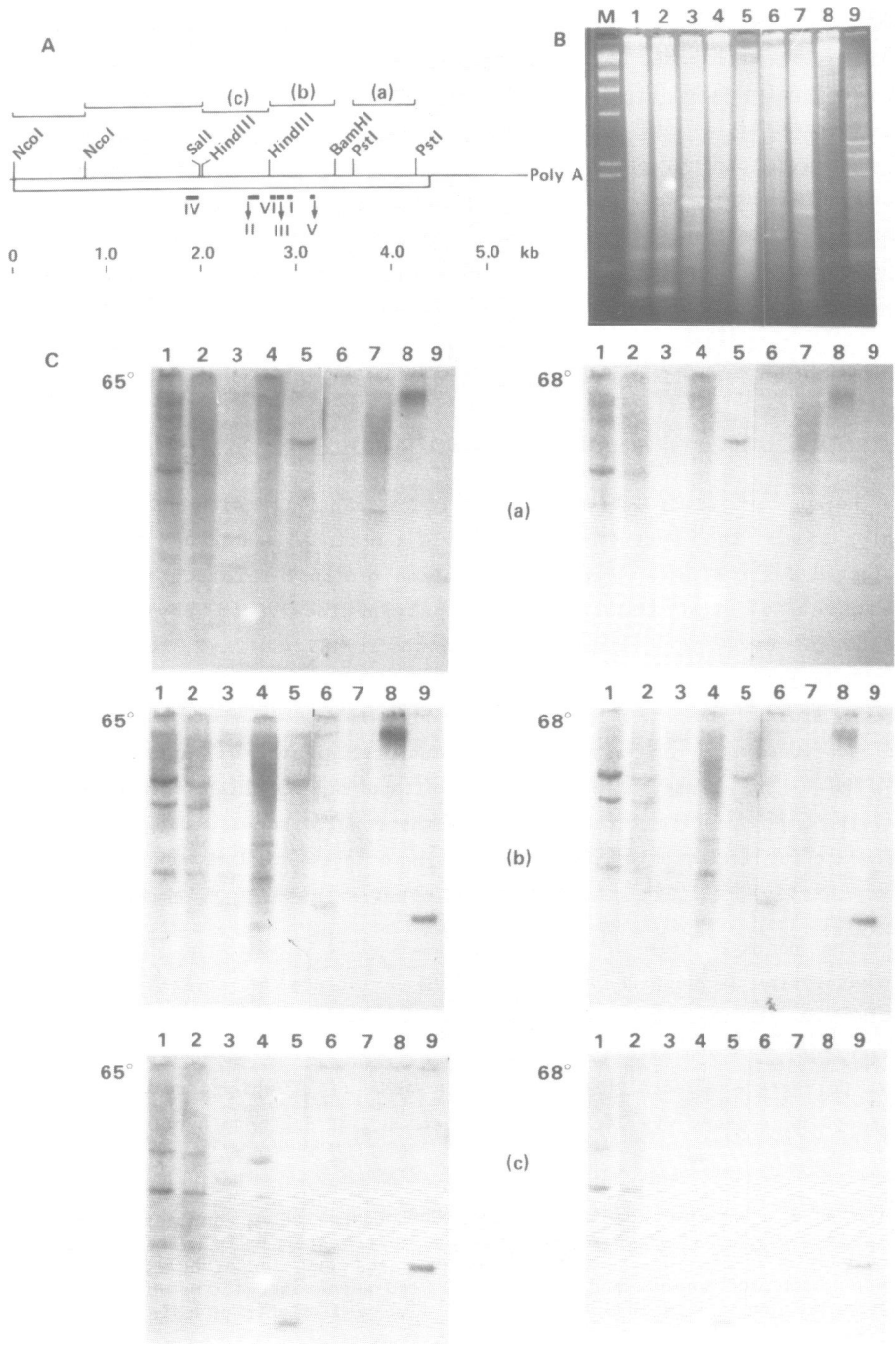
Structural conservation of the immunoreactive DNA polymerase α catalytic subunit

Because of its relatively strong cross-reactivity with other vertebrate polymerases in neutralizing assays, monoclonal antibody SJK132-20 was selected for use in an immunoprecipitation protocol to study the structural features of the immunoreactive polypeptides from phylogenetically representative cell lines. As shown in Figs. 2A-E, this antibody precipitates a unique, distinct and specific 165-180 kDa polypeptide from human, simian, rodent, avian and amphibian cell lysates above a background of polypeptides which are also equally immunoprecipitated by control non-immune P3 IgG. The preservation of this epitope on the high molecular weight catalytic polypeptide further supports the conclusion of strong evolutionary pressure for conservation of DNA polymerase α catalytic function(s).

Conservation of DNA polymerase α genetic structure among eukaryotic species

The demonstration of conserved immunogenic domains on the polymerase α catalytic polypeptide argues for the existence of conserved nucleotide sequences in the polymerase genes of phylogenetically diverse eukaryotic organisms. To evaluate this possibility, five cDNA fragments from the human DNA polymerase α catalytic polypeptide cDNA (14), which represent >90% of the coding region, Fig 3A, were used in a set of Southern hybridizations with

Figure 2. Immunoprecipitation of DNA polymerase α antigens from crude lysates of [^{35}S]-methionine-labeled vertebrate cells with neutralizing antibody SJK 132-20. Lysates were prepared and radiolabeled polypeptides were immunoadsorbed on SJK 132-20 lgG-sepharose or non-immune P3 lgG sepharose and analyzed by denaturing slab gel electrophorsis as described (10).


A
(c)
(b)
(a)
NcoI
NcoI
SalI
HindIII
HindIII
BamHI
PstI
PstI
Poly A
IV
VI
I
II
III
V
0
1.0
2.0
3.0
4.0
5.0
kb
B
M 1 2 3 4 5 6 7 8 9
C
65°
68°
(a)
(b)
(c)
1 2 3 4 5 6 7 8 9

genomic DNA samples from phylogenetically representative eukaryotic species, Fig 3B. The extent of nucleotide sequence conservation was assessed by washing the hybridized blots in a buffer of defined ionic strength under conditions of increasing stringency from 55°C to 68°C. Since the human DNA polymerase α gene was previously localized to the X chromosome (14,17), a genomic DNA sample from a human cell line (GM 1202A) containing 4 X chromosomes was included in all hybridizations as a human DNA reference sample. Starting from the 3'-end of the coding region, the Pstl/Pstl fragment hybridizes weakly with calf, rodent, Drosophila, and yeast genomic DNA and hybridizes relatively strongly with Xenopus and tobacco genomic DNA, Fig 3C(a). As shown in Fig 3C(b), the BamHl/Hind III fragment hybridizes to genomic DNA samples from calf, rodent, Xenopus, Drosophila and yeast after washes at 68°C but does not hybridize to tobacco or algae genomic DNA. Similarly, the Hind III/Hind III cDNA restriction fragment hybridizes to calf, rodent, Xenopus, Drosophila, and yeast genomic DNA after washes at 68°C but not to tobacco or algae genomic DNA, Fig 3C(c). The presence of an indistinct large size smear of signal at the top of the algae genomic DNA gel lane (Lane 8, Figs. 3C(a) and 3C(b)) is presumably due to non-specific hybridizations between the cDNA probe and an undigested portion of genomic DNA, and therefore does not represent true hybridization between the probe and algae genomic DNA.

An additional series of experiments with two human cDNA fragments which are further 5-'upstream, Sall/Ncol and Ncol/Ncol, exhibit no significant

Figure 3. Southern hybridization of human cDNA fragments with genomic DNA samples from various phylogenetic species. (A) Restriction map of human DNA polymerase α cDNA. The open box represents the coding region of human DNA polymerase α and the solid line indicates the 5' and 3' non-coding regions. The solid bars on the top labeled (a), (b) and (c) represent the corresponding cDNA fragments used for hybridization. The black bars underneath represent the designated consensus sequences (14). (B). Ethidium bromide stain of the digested genomic DNA samples from representative species. The hybridization and wash conditions are described under **Methods**. Lane M, Hind III digested lambda DNA marker; lane 1, human genomic DNA of the 4 X chromosome (49, XXXXY) cell line GM1202A; lane 2, human DNA from KB cells; lane 3, Calf thymus DNA; lane 4, murine DNA; lane 5, Xenopus DNA; lane 6, Drosophila DNA; lane 7, tobacco DNA; lane 8, green algae DNA and lane 9, yeast DNA. (C) Genomic Southern hybridization: (a) Hybridization with the Pstl/Pstl fragment of human DNA polymerase α cDNA. (b) Hybridization with the Bam Hl/Hind III cDNA fragment. (c) Hybridization with the Hind III/ Hind III cDNA fragment. The washing temperature of each blot is shown at the top left corner of the respective panel. Blots were washed under conditions of increasing stringency from 55°C to 68°C. Only the results obtained at the two highest temperatures are shown. DNA samples of each lane are as described in (B)

hybridization with lower mammalian, vertebrate, invertebrate, plant or yeast genomic DNA (data not shown). These two fragments hybridize poorly to human genomic DNA at 55°C as well. The finding that the 5'-end of human polymerase α gene comprises many small exons separated by large introns (B. E. Pearson and T. S. -F. Wang, unpublished observation), may explain the weak hybridization of these two restriction fragments with human genomic DNA. Conversely, the absence of introns in the yeast genome would explain the generally strong hybridization observed between the two human cDNA fragments (Bam HI/Hind III and Hind III/Hind III) and yeast genomic DNA. In addition, the ability of these two cDNA fragments to hybridize with genomic DNA from a variety of eukaryotic sources indicates the conservation of extensive sequence similarity in these regions of the polymerase genes.

Previous study indicated that, in the deduced amino acid sequence of the human DNA polymerase α catalytic polypeptide, there are six consensus sequences between human and human viral/bacteriophage DNA polymerases (14). Five of these consensus amino acid sequences are localized within the two most extensively cross-hybridizing restriction fragments, BamHl/Hind III and Hind III/Hind III and three of these five consensus regions are predicted nucleotide (dNTP) interacting domains (14,22-25; J.S.Gibbs, H.C.Chiou and D.M.Coen, personal communication). The results presented in Fig 3, in general, support the conclusion that these two human cDNA fragments are conserved in vertebrate, invertebrate and unicellular organisms and suggest that these sequences correspond to functional domains that are essential for DNA polymerase catalysis.

DISCUSSION

We have used four monoclonal antibodies specific for a physico-chemically and enzymologically well-defined DNA polymerase α antigen (6,10,20) to evaluate the structural conservation of DNA polymerase α and to study the molecular evolution of four unique polymerase epitopes. A comparative analysis of the structure and organization of the polymerase α gene was also performed with cDNA probes representing >90% of the coding region of the catalytic polypeptide of human DNA polymerase α (14). Data from these immunological assays and molecular genetic analyses support the conclusion there exists strong evolutionary pressures to conserve critical functional domains on the DNA polymerase α molecule.

The ability of the most cross-reactive antibody used in this study, SJK132-20, to recognize a 165-180 kDa polypeptide from species as diverse as

human and the amphibia underscores the striking evolutionary conservation of this specific epitope over a period of approximately 350 million years and suggests the critical role of this site in polymerase α catalysis. It is surprising, however, that neutralizing antibody SJK 132-20 in particular fails to recognize polymerases α from vertebrates lower than amphibia, or from any invertebate species tested, when one considers the similar polypeptide composition and the singular deoxynucleotide polymerizing mechanism of DNA polymerases from these eukaryotic organisms, as well as the sequence relatedness among the polymerase genes from species as phylogenetically distant from humans as the fungi (Results, Fig. 3). It is possible that the epitopes recognized by this (and any of our other) monoclonal antibodies do not correspond to these conserved DNA sequences. Alternatively, the primary structures of at least some of the epitopes may be encrypted within those conserved nucleotide regions, but the epitopes from the different species may exhibit different topographies in (or in proximity to) critical catalytic domains. Clarification of this issue can only be resolved by isolation of the gene and comparative analysis of the deduced protein sequence of the DNA polymerase α enzyme from appropriately representative species.

The demonstration of the ability of two consecutive cDNA fragments to cross-hybridize to distinct genomic DNA segments from species as distant from human as Drosophila and yeast underscores the strong evolutionary conservation of nucleotide sequences within the polymerase gene(s) of vertebrate, invertebrate, and unicellular organisms. Our previous study has shown that, in the deduced protein sequence from these two most cross-hybridizing segments of cDNA, there are five conserved regions which correspond to consensus amino acid sequences in the primary polypeptide structure of bacteriophage and human DNA virus polymerases (14). The demonstration of these consensus sequences among human and viral/bacteriophage DNA polymerases, and the ability of the encoding DNA sequences to cross-hybridize with vertebrate, invertebrate and unicellular fungi (yeast) genomic DNA suggests that the DNA polymerases α from these widely divergent eukaryotic species and the viral/bacteriophage polymerases constitute a class of DNA polymerizing enzymes that may have evolved from a single primordial gene.

The phylogenetic data presented in this report provide a valuable reference for investigators studying DNA replication in diverse eukaryotic systems. The results of the immunological study in this report provide

practical information to the utility of these monoclonal antibodies for immunoaffinity purifications and comparative structural studies in various eukaryotic systems. These antibodies can also be used specifically and selectively to deplete eukaryotic cell extracts of DNA polymerase α (or polymerase/primase) activity. The depleted extracts can then be fractionated and added back to purified polymerase (or polymerase/primase) fractions in defined complementation assays to isolate accessory replication factors (26). Thus far, cDNA of only two replicative eukaryotic DNA polymerases from human cells (14) and yeast (21) have been isolated. The finding of two consecutive cDNA segments of human polymerase α (BamHl/Hind III and Hind III/Hind III) having extensive sequence similarity to the corresponding genes in both vertebrate and invertebrate species also provides practical applications. These gene probes should be extremely useful to isolate cDNA clones of replicative DNA polymerases from other living species. Finally, because of the critical role of polymerase α in eukaryotic cell replication and the strict regulation of polymerase α gene expression in mitotically cycling cells (27), our results provide important guidance for the application of these antibodies and gene probes to studies of experimental carcinogenesis in diverse biological systems.

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Abbreviation: SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl/0.015 M Na-Citrate /0.5 mM EDTA

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