ISAS - INTERNATIONAL SCHOOL
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CHARACTERIZATION AND MOLECULAR CLONING OF A HUMAN DNA HELICASE IV

Thesis submitted for the Degree of

Magister Philosophiae

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CONTENTS

1.INTRODUCTION

1.1HELICASES: IMPORTANCE, ACTION AND POLARITY

Genetic information is determined by DNA which is generically present in a double stranded form. The antiparallel strands of the duplex were held together by hydrogen binding between the bases that can form pairs (A-T and G-C). For many DNA transaction such as replication, repair, recombination, transcription DNA needs to be present in a single stranded form. A class of the enzyme , the DNA helicases, enzymatically unwind DNA by disrupting the hydrogen bonds that hold the two strands together using the energy of ribonucleoside and deoxyribonucleoside 5'-triphosphate hydrolysis. Apart from DNA helicases, RNA-RNA and RNA-DNA helicases have also been identified and these have been considered to function in transcription (1) , translation (2) or RNA splicing.(3) .
The most common substrate for detecting the helicase activity is partially duplex DNA consisting of ^{32}P labeled complementary oligonucleotide annealed to M13mp19 phage ssDNA. The helicase in the presence of energy source displaces one of the strands of the duplex. The single strand can be detected by sensitivity to single-strand specific endonuclease or more often by altered mobility in electrophoresis on a nondenaturing gel.
Helicases may be classified by:
1) structural preference for the DNA substrate
2) polarity of translocation and
3) processivity

1) Some helicase can exert its activity at blunt end, whereas others require a 3' or 5' single stranded tail or even a fork like structure. Substratas commonly used to assay helicase activity are shown in figure 1:

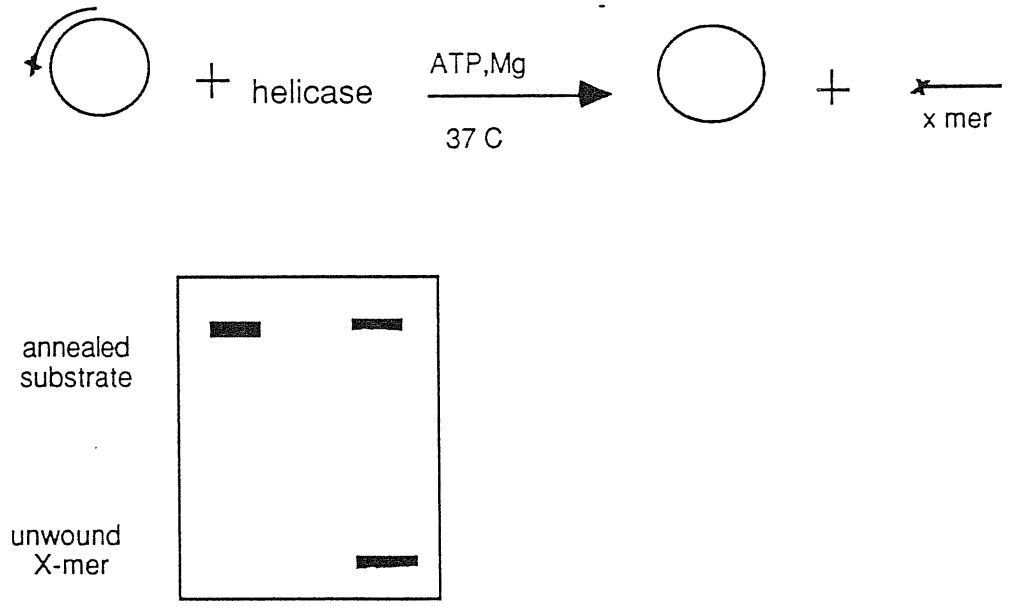


Figure 1: Sheme for measuring helicase activity. ^{32}P labeled oligonucleotide of different length is hybridized to M13ssDNA. This substrate is incubated with the sample containing helicase which unwinds X-mer oligonucleotide. In the bottom pannel the products are separated by electrophoresis in a 12% nondenaturing polyacrylamide gel.

2) Helicase can translocate in 3'-5' or 5'-3' along the ssDNA to which it binds . A single-stranded molecule with a duplex portion of different length at each of its two ends can be used to determine the polarity.

3) Helicase processivity is measured by the number of nucleotides separated during each round of helicase binding to the DNA.

DNA helicases are ubiquitous in nature. They have been isolated and described from wide variety of organism including E.coli (4) table 1, yeast, calf thymus, lily , mouse, frog and Hela cell (5) table 2.

Table 1: E.coli helicases

Helicase	*Mr (kD)*	*Direction*	*Cofactor*	*Substrate demand*	*Remarks*
Helicase I	180	5'-3'	ATP=dATP >NTPs	no tail requirment	-involment in transfering F plasmid -site-strand specific nick in oriT
Helicase II	73	3'-5'	dATP>ATP	DNA-RNA 3'ss tail at low concentration blunt end at higher	-DNA repair (excision and mismatch) -antirecombinase
Helicase III	20 (dimer)	5'-3'	ATP=dATP	partialy duplex of 500 bp	
Helicase IV	75	3'-5'	ATP=dATP >GTP	partialy duplex of 71bp	-supress the lethality of UvrD rep double mutants
Rep	68	3'-5'	ATP=dATP >GTP=dGTP	duplex of 70bp DNA-RNA high processive on the OX and M13RF	-melts RF of OX and M13 -interact probably with gpA and gp2 endonuclease -15kD cellular protein stimulate activity
Pri A	76	3'-5'	ATP=dATP	partial duplex 10-15 fold greater with DNA with PAS	-recognize PAS sequence -displace strand in primosome path -SSB dependence
dnaB	50	5'-3'	ATP>GTP= CTP	fork like structure	-active within initial replication for E.Coli oriC,phage I ori I,OX primosom -stimulation by SSB and primase
UvrAB		5'-3'	ATP=dATP	D-loops 51bp duplex requirment for short region of 5'ssDNA	-scanning of DNA for DNA damage
RecBCD	330		ATP=dATP	binds both strands during unwinding	-provide ssDNA for RecA recombination -DNA repair
Rho	50	5'-3'	ATP	RNA-DNA	-transcription termination

Table 2: Eukaryotic and viral DNA helicases

Source	*Mr (kD)*	*Direction*	*Cofactor*	*Remarks*
Virus				
SV40 T-antiger	94	3'-5'	ATP>dATP> dTTP=UTP	-interacts with DNA poly I
Polyoma T-antigen	100	3'-5'	ATP=dATP> CTP=UTP	
HSV-1 UL5/UL8/UL52	120,97, 70	5'-3'	ATP>GTP> CTP=UTP	-UL5 and UL52 required for helicase-primase activity
UL9	83	3'-5'	ATP=dATP> CTP>dCTP	-ori binding protein
BPV-1 E1	68			-ori binding protein which is stimulated by E2
AAV Rep68	68		ATP	-site and strand specific endonuclease
Yeast				
ATPase III	63		ATP>dATP	-stimulates yeast pol I
Rad 3	90	5'-3'	ATP	-function in excision repair
Rad H	134			-only sequence known
PIF1	97	5'-3'	ATP	-ssDNA dependent ATPase , mitochondrial
Lily			ATP	
Xenopus laevis	140	3'-5'	ATP=dATP	-not processive
Mouse ATPase I	58	5'-3'	ATP>dATP= dGTP=GTP	
Human				
RIP100	100	3'-5'	ATP=dATP	-interacts with RIP60 ori binding protein
Helicase I	65	3'-5'	ATP>dATP	RNA-DNA unwinding
Helicase III	46	3'-5'	ATP=dATP	-preference for fork like structure

Source	*Mr (kD)*	*Direction*	*Cofactor*	*Remarks*
Helicase IV	100	5'-3'	ATP>dATP	-substrate for cdc2
Helicase V	92	3'-5'	ATP=dATP	-copurified with HDH III
Human	72	3'-5'	ATP=dATP> CTP>dCTP	-dependent on RF-A -releases fully duplex
Human		3'-5'	ATP=dATP> CTP>dCTP	-resembles calf thymus helicase A
Calf thymus Helicase A	47	3'-5'	ATP=dATP> CTP>dCTP	-dependent on RF-A on long substrate
Helicase B	100	5'-3'	ATP=dATP	-binds to dsDNA
Helicase C	40	5'-3'	ATP=dATP>> all other	
Helicase D	100,45	5'-3'	ATP=dATP	-forms large agregates in low salt
copurified with ε	58	5'-3'	ATP>dATP> CTP	
copurified with δ	90-100	3'-5'	ATP=dATP	
Nuclear helicase I	200,170	3'-5'	ATP=dATP	
Nuclear helicase II	130,100	3'-5'	ATP=dATP> all other	

1.2 E.COLI HELICASES INVOLVED IN DNA REPLICATION

dnaB protein

dnaB protein, a hexamer of 50 kD subunits shows a helicase activity when so called preformed fork structure is used as a substrate. Helicase translocates 5'-3' direction and requires either ATP, GTP or CTP as an energy source. The protein is important for the initiation of phage oriλ and oriC DNA replication. Since it has very low affinity to DNA template inefficient oriC and oriλ binding is overcome by origin initiation protein. At oriC, the dnaC proteins form a 6:6 complex with dnaA which helps loading of dnaB into the oriC dnaA protein complex. λP dnaB complex loads dnaB into the oriλ λO complex. dnaB protein is active as a helicase at origin only upon dissociation of dnaC or λP.
E. coli SSP inhibits dnaB binding probably by competing with, while once bound it stimulate its helicase activity stabilizing unwound ssDNA.
dnaB protein acts as well at primosome assembly sites (PAS). The ϕX type preprimosome is a mobile multiprotein complex consisting of six E.coli replication proteins: PriA, PriB, PriC, dnaB, dnaC, and dnaT. Assembling starts by PriA recognition of PAS sequence. Preprimosome contains two helicases with opposite direction: PriA with 3'-5' activity and dnaB with 5'-3' activity probably to couple continous and discontinous-strand synthesis. dnaB protein separates the DNA strands to generate the fork while PriA translocating behind it, causing DNA to loop. Upon addition of primase (dnaG gene product) the primosome synthesizes multiple primers while translocating along a template (6).

Rep protein

Rep protein is required for rolling cycle replication of a number ssDNA phages: ϕX174,fd,M13,P2. After cleavage at the origin of replication by initiator endonuclease (gpA for ϕx and gp2 for M13) to generate free 3'-OH for extension by DNA polymerase, Rep melts

the replicative duplex form translocating in the 3'-5' direction migrating along the leading strand template ahead of the advancing DNA polymerase. High processivity of Rep protein depends on presence of those endonucleases suggesting there possible interaction (Rep and gpA) (7) . A small protein of 15kD has been purified as a cellular counterpart of phage proteins which stimulates helicase but not ATPase activity (8)

1.3 E.COLI HELICASES THAT FUNCTION IN TRANSCRIPTION AND REPAIR

Rho protein

The Rho protein, a hexamer of 50kD subunits catalyzed ATP driven melting of RNA-DNA hybrid. The enzyme translocates 5'-3' direction. Each subunits contains three functional domains: N-terminal contains RNA binding domain, ATP binding site locating in the central portion and subunits interaction site in the C-terminal region (9).

Transcription termination in E.coli occurs either at intrinsic or Rho-dependent termination site. , Rho is required for release of RNA transcript. Termination requires thermodynamic destabilization of the transcription complex probably trough ATP-dependent RNA-DNA helicase activity of Rho protein. Rho protein binds as a hexamer to Rho-dependent termination site and translocates along the RNA by one "dimer length" (22-24 nt) toward the 3'end of the RNA chain. When encounters the double stranded RNA-DNA hybrid a portion of correctly positioned Rho binding site can interact with transiently exposed segment of RNA , appearing as a consequence of destabilization induced by vicinity of Rho, and initiate separation of RNA-DNA hybrid (10).

Helicases that function in repair

The nucleotide excision repair in E.Coli starts when two molecules of UvrA dimerize in the presence of ATP and complex with one molecule of UvrB (11). The complex then bind DNA by very weak 5'-3' helicase activity is capable of unwinding short stretches of duplex DNA in the presence of ATP or dATP. In this way it may translocates along one of the strands of the helix sccaning it for distorting region (12). Once the lesion has been detected translocation stops and UvrB is captured onto the DNA inducing specific DNA conformation. The UvrA molecules dissociate allowing UvrC to bind (figure 2) (11).

Helicase II (UvrD), together with DNA polymerase I act in releasing of UvrBC incised 12-13 nt fragment containing lesion by melting at the nick. Helicase is also important for UvrABC turnover (13).

Excision of the incised strand in methyl directed mismatch repair is also dependent on helicase II (6).

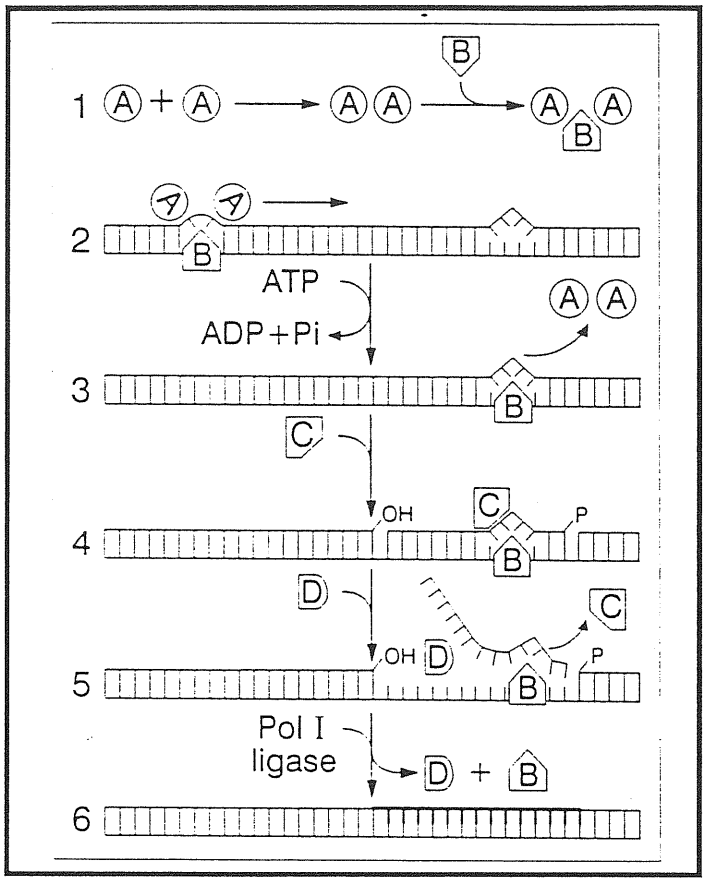


Figure 2: Model for the reaction mechanism of E.Coli nucleotide excision repair: 1.formation of UvrAB heterotrimer, 2.UvrAB scanning of DNA, 3. UvrB capture and releasing of UvrA, 4. UvrC binding and incision of oligonucleotide containing lesion, 5. excision of oligonucleotide by UvrD, 6. DNA synthesis and ligation

1.4 EUKARYOTIC HELICASES

1.4.1 VIRAL HELICASES

Large T antigen

SV 40 DNA replication provided a nice model system for studing cellular DNA replication since it requires participation of only a single viral protein SV 40 large T-antigen.
Large T-antigen is a multifunctional 82 kD phosphoprotein which shows ATPase and helicase activity. It binds specifically and with high affinity to the viral replication origin. In the presence of ATP T-antigen monomers assemble as a hexamer on each half of the origin locally unwinding the DNA. In the presence of RF-A and topoisomerase it translocates 3'-5' direction generating two replication forks. The formation of initiation complex depends on DNA polymerase α/primase binding leading to RNA primering. The structure could be then elongated by highly processive DNA polymerase δ , RF-C and PCNA. The interaction of large T-antigen with DNA polymerase δ indicates its possible role in elongation step (14).
Biochemical activities of T-antigen are modulated by phosphorylation of at least eight serine residues located near the amino terminus and threonine residues located near the carboxyl terminus. Casein kinase I (CKI) phosphorylates T-antigen on serine residues inhibiting origin specific unwinding and in vitro SV40 DNA replication. The intrinsic helicase activity was not impaired (15). Erdile et al, using PP2A phosphatase which removes the inhibitory phosphates from serine residues, have demontrated that the major effect of dephosphorylation was to increase the formation of double hexamers on the origin relative to a single hexamer. Probably the phosphorylation allows a favorable protein-protein interaction between two hexamers which could be critical for initial opening of origin (14).

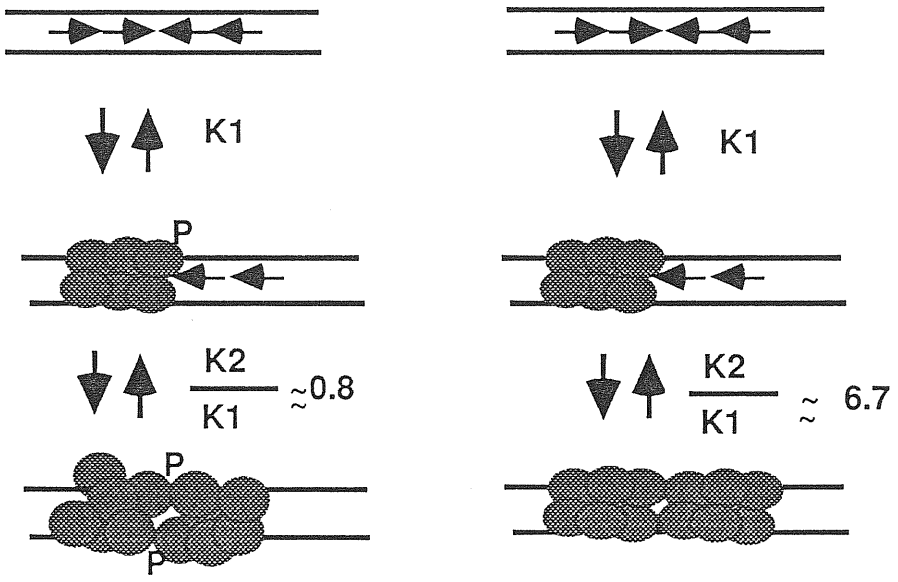


Figure 3: Diagram of the ratio of the apparent association constant of the second hexamer bound to the origin to that of the first one

Recently it has been shown (unpublished observation) that phosphorylation at Thr 124 by cdc2 enhances its ability to initiate DNA synthesis. A mutant T-antigen (Thr124-Ala) failed to replicate SV 40 DNA. The mutant binds specifically to the viral origin as a hexamer but it is defective in unwinding of circular plasmid DNA containing the SV40 origin suggesting its deficiency in origin unwinding (16).
Hurwitz et al. have demonstrated that direct association of T-antigen with cyclin A prevents kinase complex to phosphorylate histone H1 in vitro (unpublished observation)(16). Cyclin A cdk2 complex appears in the S phase of the cell cycle. Interaction with large T-antigen or cellular counterpart modulate activity of the kinase which might preferentially phosphorylate T-antigen and other substrates important for DNA synthesis.

Interaction of p53 with large T-antigen

p53 appears to have a tumor supressor function arresting cell at the G1/S border. There is some indication that it could be involved in the regulation of DNA synthesis.
Binding of p53 with SV40 T-antigen inhibits it helicase activity blocking its ability to mediate DNA synthesis. Binding site for p53 is found to be in a very vicinity of viral replication origin (17).

Herpes simplex virus DNA helicase-primase complex

Herpes simplex virus 1 (HSV1) encodes seven proteins that are required to initiate and sustain DNA replication at any of three origin of virus DNA replication (ori_L and diploid ori_S) (18).
Three of this protein encoded by the UL5, UL8 and UL52 genes form a heterotrimer with both DNA helicase and DNA primase activities (19). Lehman et al. demonstrated that the subassembly of only UL5/UL52 subunits exibit all the enzymatic activities: ATPase, DNA helicase and primase. Although the UL5 protein bears sequence homology to the nucleoside triphosphate binding domain and conserved DNA helicase motifs it does not show DNA helicase and ATPase activity. The association with UL52 is abolutly required (20).
UL8 is required for viral DNA replication probably through stabilizing the association between nascent oligoribonucleotide primers and template DNA thus increasing the efficiency of primer utilization. The protein exist as a monomer in solution and it does not bind to DNA (21). This is the property similar to proliferating cell nuclear antigen (PCNA) which is important for the processivity of DNA polymerase δ but it does not bind to DNA in the absence of another protein: replication factor C (RF-C) (22).
UL9 is an origin binding protein showing an ATPase and DNA helicase activity (23).

1.4.2 DNA HELICASES INVOLVED IN NER

The yeast nucleotide excision repair gene Rad3 is a single stranded DNA-dependent ATPase and also acts as a DNA helicase on partially duplex DNA. Rad3 helicase activity is inhibit upon different damaged of DNA strand to which it binds and presumably translocates. The sensitivity of Rad3 helicase activity to DNA strand specific inhibition and the consequent formation of stable Rad3-DNA complexes may serve in the very beginning of the NER: searching and locating the sites of DNA damaged (24).

Mammalian NER genes have been isolated either by transfection of genomic DNA into UV-sensitive rodent mutant (ERCC gene) (25) or by transfection of extrachromosomally replicating vector into a human cell from the patients with human repair disorder Xeroderma Pigmentosum (XPC gene) (26).
Deduced amino acid sequence of some of those genes shows the existence of seven consecutive domains well conserved between superfamilies of DNA and RNA helicases. This is not surprising since the helicase activity is important in two steps of excision pathway : scaning for the DNA damaged and displacement of incised oligonucleotide containing lesion (27).
Recently it was found that the product of the ERCC3 gene which corrects the repair deficiency of XP-B, characterized by overall genome deficiency and deficiency in preferentally repair of actively transcribed genes, seems to be the larger subunits of basal transcription factor TFIIH (28).

1.4.3 IN VIVO FUNCTION OF OTHER CELLULAR HELICASES

Replication of the Chinese hamster dihydrofolate reductase gene (DHFR) initiates near a fragment of stably bent DNA. RIP 60 polypeptide has been found to contain origin-specific activity while the copurified helicase activity has been address to 100kD protein called RIP 100 (29).

A mismatch-binding protein of 160 and 100 kd have been purified from Hela nuclear extract. Both of them bind specifically to oligonucleotide duplexes containing G/T mismatches and show an associated DNA helicase activity (30).
eIF-4A is translation initiation factor capable of unwinding RNA secondary structure present at the 5'end of a mRNA transcript. The reaction is dependent on ATP and it is stimulated by eIF-4B. The helix unwinding step is rate limiting if the RNA is masked by stable secondary structure (31). Recently a SSL2 gene from S.Cerevisiae has been isolated as a postranscriptional supressor of HIS4 mutant allele that encodes a stem loop structure which apparently blocks the ability of ribosome to bind and scan mRNA. SSL2 gene product belongs to the growing family of putative helicases (32).

1.4.4 A DNA HELICASES FROM HELA CELLS

Tuteja et al. (33,34,35,36) have initiated the purification and characterization of the DNA helicases from Hela cells. Up to now four different helicases have been isolated and characterized. HDH I, HDH III, HDH V have been isolated through differential chromatographic fractionation. The fractionation depends on there behaviour on weak acidic cation exchanger Bio-Rex. HDH III (46 kD) and HDH V (92 kD) bind to the column while HDH I does not. HDH III and V elute together at 0.8 M NaCl. The helicases were separated one from the other by Heparin Sepharose column after elution with 0.45 M KCl for HDH V and with 0.57 M KCl for HDH III. HDH III has then been purified to homogeneity performing anion exchanger Q-Sepharose chromatography, ssDNA affinity column and Mono Q chromatography. HDH V on the contrary was purified through Q-Sepharose to which it does not bind and ssDNA affinity column. The most distinctive properties of HDH III helicase was it preference for fork like-structure. This implied it possible role: involment in the advancement of the growing fork or through binding to partially unwound DNA, as a consequence of chemical damages or mismatching, in DNA repair. HDH V differs from the other human helicases being present in extremely low abundance, being highly

active and by using as well the small linear DNA as a substrate. After being passed through Bio-Rex 70 column, HDH I (65 kD) was subsequently purified on DEAE-Sephacel column followed by ssDNA affinity column. HDH IV was present in the supernatant upon precipitation with 35% [w/v] ammonium sulfate. Subsequent purification steps were written elsewhere (the aim of the work).

1.5 MECHANISM OF DNA UNWINDING

Two models for mechanism of helicase DNA unwinding has been proposed: passive or active, depending of whether the helicase only stabilizes the resulting ssDNA or it participates in the unwinding event.

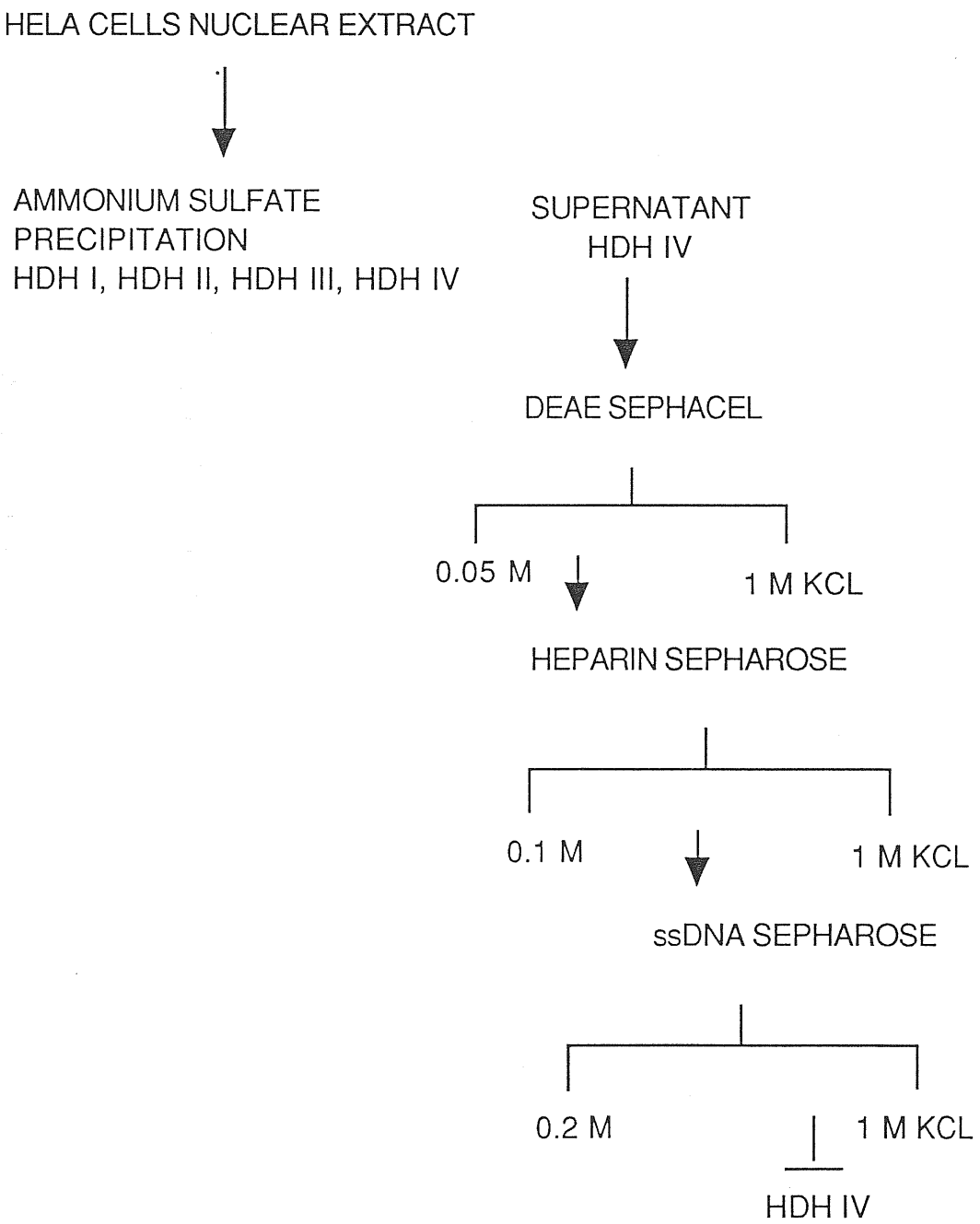
Passive mechanism requires the helicase to possess only one DNA binding site to interact with ssDNA and the helicase activity to translocate unidirectionally along the ssDNA toward the duplex. ssDNA can become available through transient unwinding of the duplex caused by thermal fluctuation at the ss/dsDNA junction or upon binding of different DNA binding proteins causing the locally melting of duplex DNA.

Active mechanism requires the helicase to possess at least two binding sites. In the rolling mechanism represented by Rep protein helicase interacts directly with dsDNA at the junction and actively destabilized some number of base pairs through conformational changes of helicase driven by NTP binding and hydrolysis. The model requires that the helicase is able to bind both ds and ssDNA and both simultaneously in at least one of intermediate step.

In the second model helicase binds simultaneously to both ss strand at the ss/dsDNA junction and unwinds the duplex through its NTP-dependent conformational change (37).

THE AIM OF THE WORK

Human helicase IV has been purified to homogeneity from HeLa cells and characterized (33). The purification was performed as following:



The apparent molecular weight is 100 kDa. The helicase activity was measured by assaying the unwinding of ^{32}P labeled oligonucleotide annealed to M13 ssDNA. It requires divalent cation for activity (Mg^{2+} = Mn^{2+} =Zn^{2+}) and the hydrolysis of only ATP or dATP. The enzyme unwinds DNA by moving in the 5'-3' direction allong the bound strand.

As all helicase requires energy to exert their activity , we developed ssDNA dependent ATP-ase assay.

In order to obtain more information about the role of HDH IV helicase we also started the isolation and characterization of HDH IV gene.

2.MATERIALS AND METHODS

DNA dependent ATPase assay-We modified the original method described by Hubscher and Stadler (38). The reaction mixture (10µl) contained 20mM Tris-HCl (pH 8.5), 8 mM $MgCl_2$, 30 mM KCl, 4% (w/v) sucrose, 80mg/ml BSA, 1mM ATP, 166 nCi [^{32}P] ATP (5000Ci/mmol), 1µg ssDNA from HeLa cells and ~ 100ng of pure HDH IV. Assay was performed in presence or absence of ssDNA. The mixture was incubated for 30 min at 37°C and the reaction was stopped by chilling to 0°C. 1 µl of the mixture was spotted to a polyethyleneimine-cellulose thin-layer strip (0.6X6 cm), and ascending chromatography was performed in 0.5M LiCl, 1 M formic acid at room temperature for about 15 min. The strip was dried and exposed to Amersham hyperfilm to identify the radioactive spots of ATP and Pi. For quantitation these spots were cut and counted.

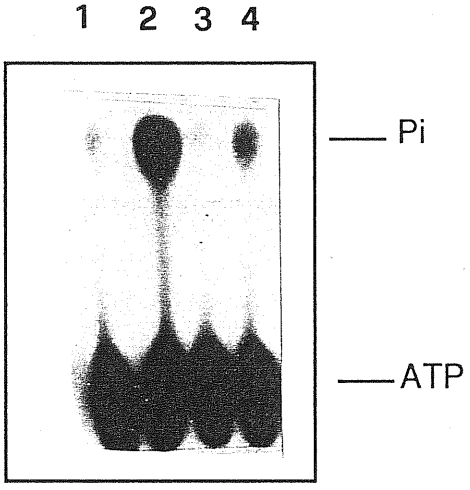


Figure 4: Autoradiography of ATPase assay. Line 1: 0 min in the presence of 1µg ssDNA and helicase ; line 2: 30 min in the presence of 1µg of ssDNA and helicase; line 3: 0 min in the presence of helicase and in the absence of 1µg ssDNA; line 4 : 30 min in the presence of helicase and in the absence of 1µg ssDNA

Antibody purification on protein A column

Rabbit polyclonal antibodies have high-affinity for protein A and can be purified on protein A columns (39).
The pH of serum was adjusted to pH 8 by adding 1/10 volume of 1.0M Tris (pH8). The serum is passed through a protein A bead column. Column binds approximately 10-20 mg of antibody per ml of wet beads. The beads are washed with 10 column volumes of 100 mM Tris (pH8) followed by 10 column volumes of 10.mM Tris (pH8). Bounded antibody are eluted with 100 mM glycine (pH3). Buffer is added stepwise, approximately 500µl and eluate is collected in eppendorf tube containing 50µl of 1 M Tris (pH8). Immunoglobulin-containing fractions is identified by Bradford dye binding spot test using Bio Rad protein assay.

Removal of anti-E.Coli antibodies by affinity chromatography

E.coli protein isolation

This method is adapted from de Wet et al (1984). A single colony of λ1090 strain was grown for at least five hours in 2X2 ml of LB and 2µl of ampicilin.at 37°. Cells were transfered into 2X200ml LB and 200µl ampicilin and grown overnight at 37°. The bacteria were recovered by centrifugation at 8000 rpm in GS3 Sorwall rotor at +4 for 20 min. The medium was poured out and the centrifuge tube were standed in an inverted position to allow the traces of medium to drain away. Pellets were resuspended in 20 ml of Na borat (pH8),1M NaCl. Cell suspensions were put together and incubated with 80 mg of lysozyme in room temperature for 20 min. and upon addition of 0.4mg of DNase and 40µl Triton X-100 was incubated at + 4°C around one hour (until the turbidity clears and the viscosity decreases). Bacterial lysate were centrifuged at 7000 rpm for 20 min using Sorwall SS34 rotor. In the supernatant protein concentration has been estimated by Bradford test.

Dialysis

Tubing were cut into a pieces of required length and boiled for 10 min in a large volume of 2% sodium bicarbonate and 1mM EDTA. The tubing were rinsed troughtly in distilled water, boiled for 10 min., cooled and store at +4°C.
Before use tubes were washed inside out with distilled water and than with coupling buffer (HCO3). Around 20 ml of E. Coli lysate is dialysed against 2 liters of coupling buffer. Dialysis has been performed for four hours (changing buffer every hour) at +4°C with magnetic stirer.
To rid of denaturated proteins and traces, centrifuge has been performed at 15000 rpm (SS34 rotor).

Coupling of the E.coli proteins

Resin were prepared according to Pharmacia instruction.
4 g of freeze dried powder is swolen for 15 min in ~80 ml of 1mM HCl and washed on a sintered -glass funnel (G3 porosity) with the same solution (approx. 200ml per g powder). The gel was than washed with coupling buffer , immediately transfered to a solution of ligand (5mg protein per ml of gel) and left to rotate overnight at +4°C. Coupling efficiency was estimated by protein quantitation. Excess of ligand was wash away with coupling buffer (250ml).
Remaing active group was washed with 1M ethanolamin pH9 and put for 2 hours in the same solution.
After that , the resin was washed in a sintered-glass funnel successively with: 100 ml of 0.1M Na-bicarbonate (pH8.3) 100 ml of 0.1M acetat buffer (pH8) containg 0.5 M NaCl, 100 ml of 0.1Tris buffer (pH8) containg 1M NaCl).
All the buffers contained protease inhibitors: 1mM DTT, 0.1 mM PMSF, 1mM sodium metabisilfite.
Before use the resin was eqilibrated in Tris buffer saline (TBS).

Affinity chromatography

1ml of Sepharose 4B coupled to E.coli has been mixed with 1 mg of IgG (previously purified on Protein A Sepharose column) and incubated overnight at +4°C on rotating wheel.
The slurry was loaded on Econo- colum^{R} (Bio Rad) and the antibody were recovered by washing with TBS. The purification has been performed three times and antibody were tested by western blotting.

Western blotting

Proteins were separated by sodium dodecyl sulfate - polyacrilamide gel electrophoresis (SDS-PAGE) described by Laemmly (38) and transferred to a nitrocellulose filter by semi dried electroblotting method for 1 hour. The filter was incubated in 10% milk-TBS-0.05 % Tween 20 for 1 hour at 37°C. Incubation with purified antibodyu was performed in 5% milk-TBST overnight at room temperature. The filter was then washed in 5% milk TBST and incubated in 5% milk TBST with alkaline phosphatase-conjugated goat anty-rabbit antibody diluted 1:3000 for 2 hours at room temperature. After successively washing with 5% milk-TBST, TBST, TBS, bound antibodies were revealed using BCIP/NBT colour development solution (Bio Rad) in AP buffer (100 mM Tris pH9.5; 10mM NaCl; 5mM $MgCl_2$).

Library screening

Expression, λgt11 and λzap libraries (Clontech laboratories) have been used for immunological screening. E.coli λ090 strain has been used for λgt11 library screening while XL-1 blue strain for λzap library screening. A bacterial (λ1090 or XL-1) colony has been inoculated into 3 ml of LB medium containing 0.2% maltose and ampicillin or tetracycline respectively. The culture were grown overnight at 37°C on rotating wheel. The cells were centrifuged at

1000g for 5 minutes and resuspended in 10mM $MgSO_4$ to appropriate density (l1090 OD_{600}~2;XL-1 OD_{600}~1)
200µl of the plating bacteria were mixed with diluted bacteriophage λ expression libraries in a sterile tubes and incubated for 20 min at 37°C. 3.5 ml of molten top agar containing 10 mM $MgSO_4$ has been added to each tube and poured to previously dried LB agar plates. The infected plates were incubated for 3.5 hours at 42°C. The plates were then overlayed with numbered nitrocellulose filters previously soaked into a solution of 10 mM isopropylthio-β-D-galactoside (IPTG) and incubated for another 3.5 hours or ~5 hours for λZap. The filters were washed in TBST (10mM Tris pH7.5; 150mM NaCl; 0.05% Tween 20) for 20 min and incubated in 5% milk-TBST for 1 hour at room temperature. Incubation with purified antibody 1:2000 diluted in 5% milk-TBST was performed overnight at room temperature. Further steps were performed as for Western blotting.

λ DNA preparation by CTAB method (39)

200µl of λ1090 plating cells were infected with 200µl of λ phage in SM buffer and absorbed for 20 min at 37°C. Upon addition of 3.5 ml of top agarose the mixture were poured onto a LB plates and incubated at 37°C until the plaques were almoust confluent. 3 ml of Tris phage buffer (10 mM Tris-HCl PH7.5, 10mM MgCl2,20mM NaCl) was added and left for 4 hours at +4°C with gentle agitation. The lysate was collected , 1/50 of volume of chloroform was added and the mixture were centrifuged for 5 sec at 5000g. To the lysate DNase was added to a final concentration of 20µg /ml, leaved at room temperature for 15 min. The lysate was then mixed for 15 min on rotating wheel after an equal volume of DE52 and gelatin at final concentration of 50µg/ml has been added. The resin was spun down twice for 1 min and 20mM EDTA and 50µg/ml proteinase K (final concentration) were added to the supernatant and incubated for 15 min at 45°C. CTAB is added to a final concentration of 0.1%. The tube is heated 3 min at 68°C, cooled down on ice for 5 min and centrifuged at 8000g. The pellet were redissolved in 1/5 volume,

with respect to the starting lysate of 1.2M NaCl and ethanol precipitated.

Polymerase chain reaction (PCR)-amplification of cDNA insert

1µl of the phage DNA was used in a PCR in a final volume of 50 µl containing 400µl each of the 4 deoxyribonucleoside triphosphate and 200ng of each λ primers in 50 mM KCl, 25mM Tris-HCl, 2mM MgCl2, 1mM DTT 2.5 units of Taq DNA polymerase (Boehringer). DNA has been denaturated for 5 min at 94°C and then PCR was carried out at 94°C for 1min, 52°C for 1min and 30 sec and 72°C for 2 min for a total of 30 cycles. Extension was performed at 72°C for another 7min. 25 µl of the PCR was analyzed and cut from 0.8% agarose gel. cDNA insert from agarose gel has been eluted with geneclean.

Colony hybridization

DNA labeling - DNA probe (50ng) was radioactevely labeled by the random primed DNA labeling method (Pharmacia/LKB). DNA was denaturated by heating at 94°C for 3 min, and then chilled on ice. To denaturated DNA the following was added: 10 µl of reagent mixture (buffered aqueous solution containing dATP,dGTP,dTTP, and random hexadeoxyribonucleotides), 5 µl (50mCi) of [α-^{32}P] dCTP (specific activity 3000 Ci/mmol), 1µl Klenow enzyme (5-10 units/µl). Final volume was adjusted to 50 µl with redistilled water and the reaction tube were incubated at 37°C for 1 hour. The reaction was stopped adding 5 µl of 0.2 M EDTA (PH8) and the radiolabeled probe was purified from unincorporated nucleotides by gel-filtration cromatography using Sephadex G-50 (Pharmacia/LKB).
Probe was then used for colony hybridization to identified E.coli recombinant clones harbouring pUC19 plasmid or for screening purpose.

Colony hybridization was carried out according to the method of Tuteja, et al (40). The transformed bacterial cell were replica plated onto a nylon membrane (Hybond-N+,Amersham,UK) previously marked with a pencil.

Cells were lysated as described by Grunstein and Hogness (41). Nitrocellulose filters were placed with colony side up on the denaturating solution (0.5M NaOH, 1.5M NaCl) impregnated Whatman 3MM paper for 5 min and then transfered to 3MM paper saturated with neutralizing solution (1.5M NaCl, 1M Tris-Cl PH8) and left for another 5 min. Filter were then washed with 2XSSPE (20XSSPE: 3.6M NaCl, 0.2M Na phosphate , 0.02M EDTA PH7.7). The DNA was fixed by baking for 90 min at 80°C in a vaccum oven.

Prior to hybridization the filters were washed twice in high salt solution (0.05 M Tris, 100 mM EDTA, 0.1% SDS and 0.9 M NaCl) at 45°C for 15 min to rid of bacteria and then prehybridized for 4 hours at 65°C in solution containing 3XSSC (1X SSC is 0.15 M NaCl, 15mM Na citrate), 2X Denhardt's solution, denaturated salmon sperm DNA (300µg/ml), and 0.2%SDS. Hybridization was carried out overnight with α -^{32}P dCTP labeled probe in a solution containing 6XSSC, 4XDenhardt's solution, denaturated salmon sperm DNA (300ml/ml), 40 mM Tris-HCl (PH7.5), and 0.2%SDS at 60°C. Ater hybridization the blots were washed successively twice for 20 min each in 2XSSC, 0.1%SDS at 57°C and once for 20 min in 0.3 SSC, 0.1%SDS at 55°C. The blots were then exposed to Kodak X-O MAT-AR with an intensifying screen at -80°C.

Transformation of E.coli DH5α cells

E. coli competent cells were prepared following $CaCl_2$-heat shock method described by Sambrok et al (42).
10 µl of cDNA amplified by PCR were blunt ended in reaction mixture containing 5 mM dNTPs, nick tranlation buffer (0.5M Tris-HCl (PH7.2), 0.1M $MgSO_4$, 1mM DTT and 500 µg/ml BSA), 2µl of Klenow fragment (2U/µl) at 37°C for 30 min. The enzyme was inactivated by cooling at 70°C for 10 min.
The phosphorylation was performed in the total volume of above reaction mixture in the presence of 10 mM ATP, nick translation buffer and T4 kinase (10U/µl) and incubated at 37°C for 30 min. The mixture was extracted with phenol-chloroform and DNA was precipitated with ethanol.
After checking the yield on 1% agarose the ligation was performed. Around 200 ng of blunt ended and phosphorylated cDNA was ligated with~ 20 ng of pUC19 previously blunt ended with Sma I enzyme digestion and dephosphorylation with calf intestinal alkaline phsosphatase and incubated overnight at 16°C.
5 µl aliquot was introduced into 50 µl of competent cells and incubated on ice for 30 min, then heat shocked at 42°C for 2 min and rapidly transfered on ice for 1-2 min. 100 µl of LB was added and incubated at 37°C for 30 min to allow bacteria to recover and to express the antibiotic resistant marker encoded by the plasmid. The transformed cells were gently spreaded over the surface of the agar plate in the presence of 40 µl IPTG (100mM) and 20 µl XGal (20 mg/ml and incubated overnight at 37°C.

DNA sequencing

Small scale preparation of plasmid DNA

The protocol is modification of the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). A single bacterial colony is transfered into 2ml of LB medium containing amphicilin and incubated overnight at 37°C. 1.5 ml of the culture was centrifuged at 12000 g fot 30 sec. at +4°C and pellet resuspendent by vigorous vortexing in 100 µl of ice cold solution I (50 mM glucose, 25mM Tris-HCl (PH 8) ,10mM EDTA (PH8)). Freshly prepared solution II (0.2M NaOH and 1% SDS) was added, the contents carefuly mixed, and incubated on ice. After addition of solution III (3M potassium acetate and 5M glacial acetic acid) the tubes were incubated on ice for 45 min. After twice centrifugation at 12000 g for 5 min at +4°C and supernatant recovering, RNAse was added at final concentration of 150 µg/ml and incubated for 1 hour at 37°C. Phenol-isoamyl-chloroform and chloroform extraction was performed and DNA was precipitated with 25% PEG containing 2.5 M NaCl.

Dna sequencing

8 µl of template DNA (1.5-2µg) is denaturated with 2µl of 2M NaOH for 10 min in room temperature. After denaturation, a Sephacryl S-400 spun column is used to remove any contaminants and NaOH. DNA was sequenced using T7 Sequencing™ kit according to Pharmacia instruction ~10 ng of primer was annealed to the template at 37°C for 20 min. Enzyme catalyzed extension of this primer is initiated using all four deoxynucleotides among which dCTP was [α-^{32}P] labeled. The labelling reaction was done for 5 min at room temperature. The extension was terminated by addition of single prewarmed dideoxynucleotide to each of the four deoxynucleotides and incubate at 37°C for 5 min. After addition of stop solution, an aliquot ~ 2µl was heated for 2 min at 95°C and immediately loaded on 6% sequencing gel. Gel was fixed in 10% acetic acid and 10%

methanol solution and exposed overnight at room temperature to Amersham X-ray film.

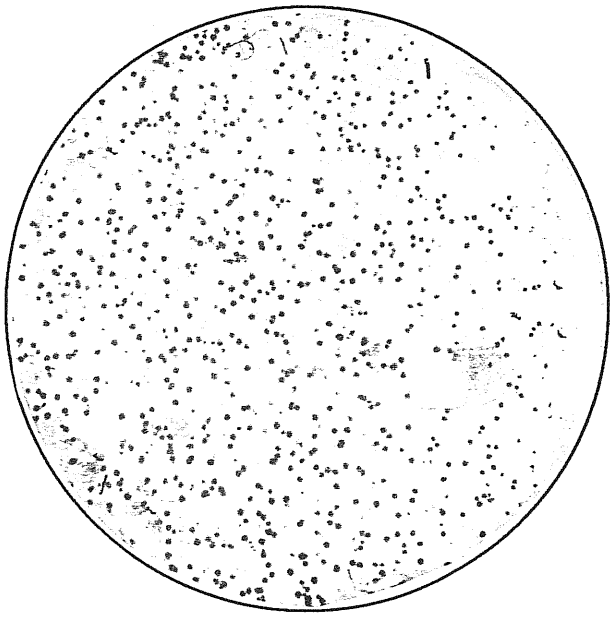
3.RESULTS

3.1. ANTIBODY SCREENING OF EXPRESSION LIBRARY

Polyclonal rabbit antiserum against HDH IV helicase has been once purified on Protein A Sepharose and used in 1:2000 dilution for screening of Hela cDNA libraries: λ gt11 and λ ZAP.

As the background was very strong (figure 5) we performed additional purification on affinity column of E.coli proteins coupled to CN-Br activated Sepharose 4B. In this way the background has been reduced significantly (figure 6).

Figure 5: Hybond C-extra filter upon screening with Potein A Sepharose purified antibody



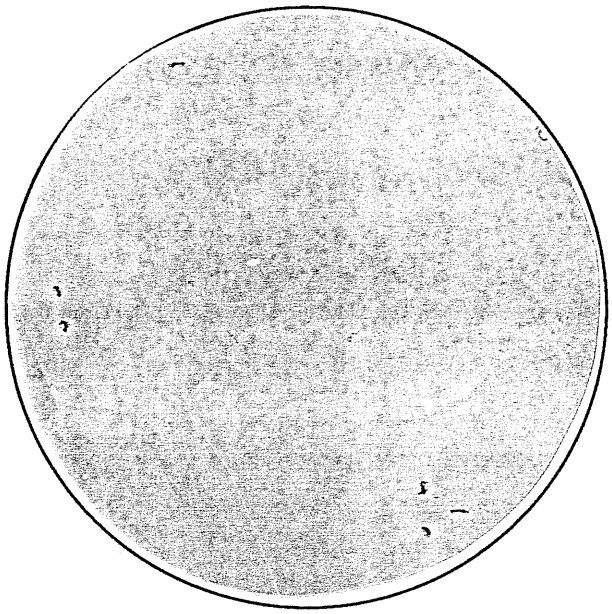


Figure 6: Hybond C-extra filter upon screening with affinity column purified antibody

The antibody has been also tested by western blot (figure7).

After screening about 3X105 recombinants, three positive clones no. 50,173 and 184 were identified and plaque purified (figure 8,9,10).The purified λ DNA from these positive clones was digested with EcoRI and no digestion product were observed. For this reason the DNA was digested with two enzymes in the polilinker region flanking the insert. Analysis of these digest revealed that clone no 50 , 173 and 184 contained insert of 1kb, 0.7 kb and 1kb respectively.

In order to characterize these clones cDNA insert from λ phage were subcloned into a plasmid by PCR amplification and blunt end ligation.

All the clones were amplified by PCR, blunt ended with Klenow fragment, phosphorylated with T4 kinase, and cloned into Sma I digested and dephosphorylated pUC19 vector.

Transformed clones were detected by hybridization with random primed labeled insert (fig 11) and further characterized by sequencing

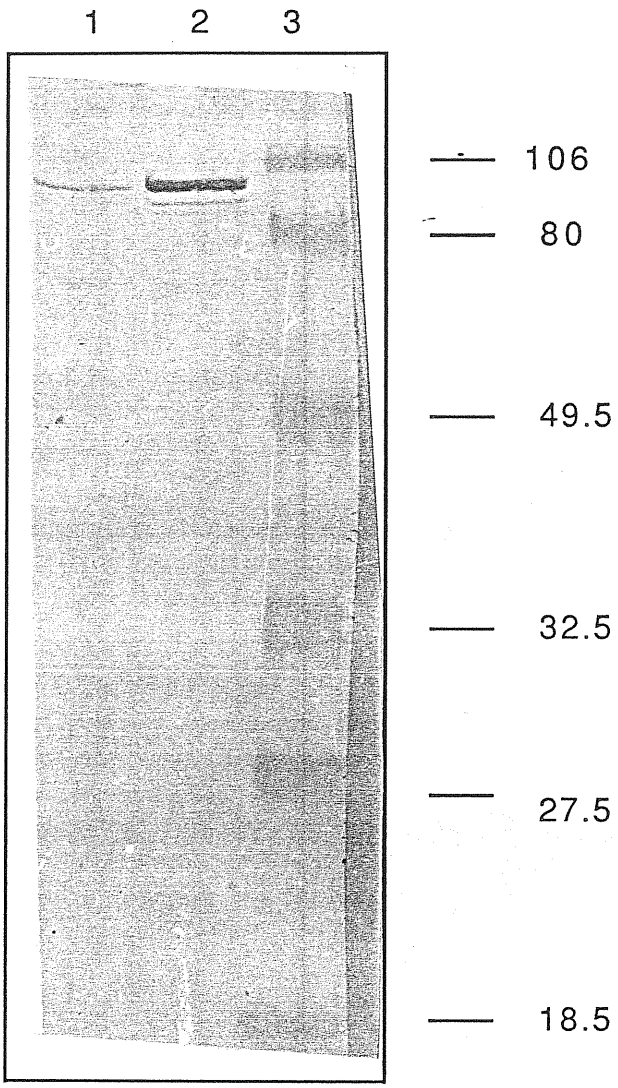


Figure 7: Western blot with antibody against helicase IV purified on affinity column. Lane 1 : pure helicase IV(50 ng); lane 2: nuclear extract; (20 ng) lane 3: low range prestained marker

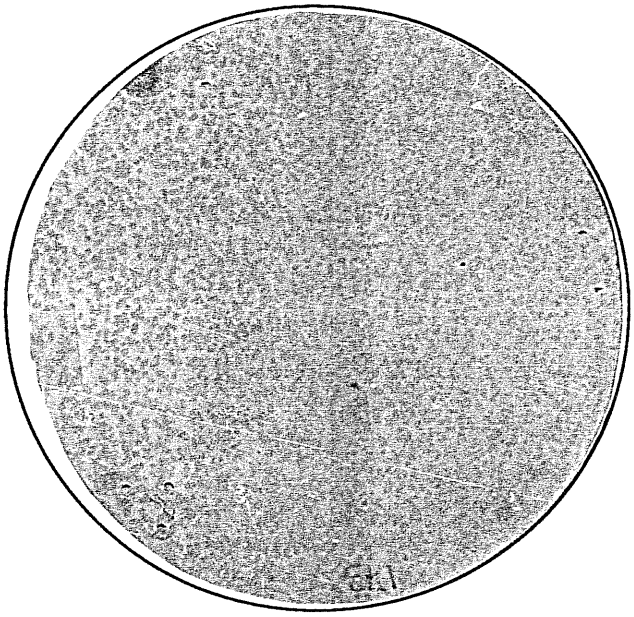


Figure 8: Hybond C-extra filter with first positive clone no.50 after first screening

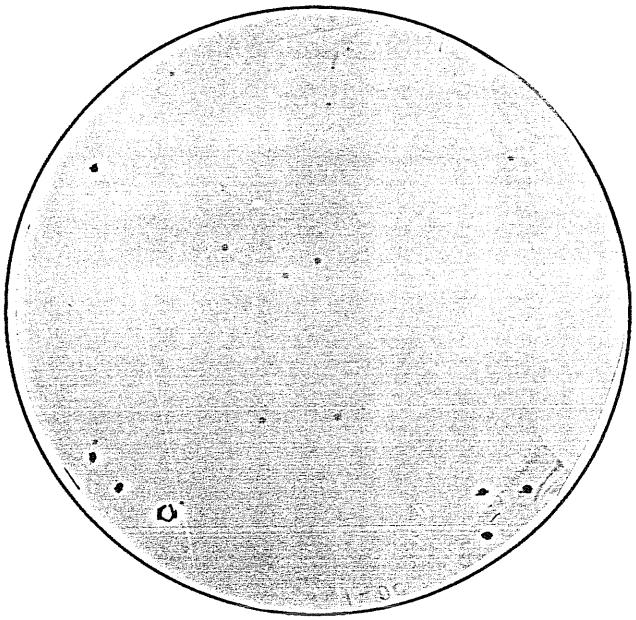


Figure 9: Hybond C-extra filter with positive clone no.50 after second screening

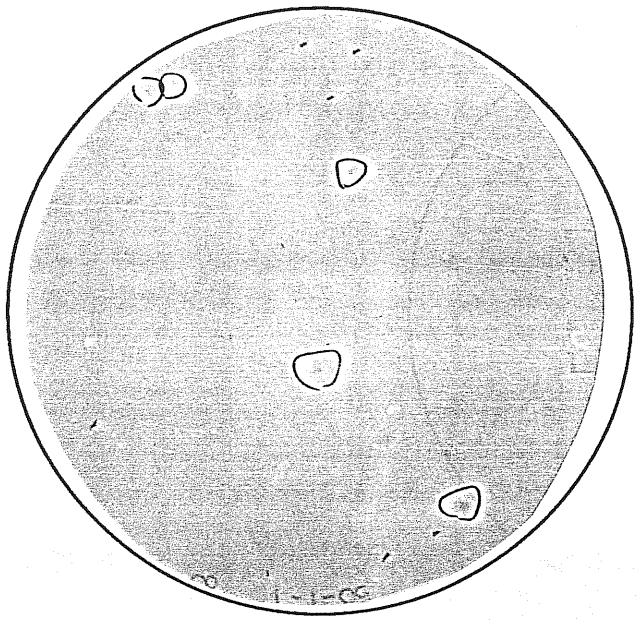


Figure 10: Hybond C-extra filter with positive clone no.50 after third screening

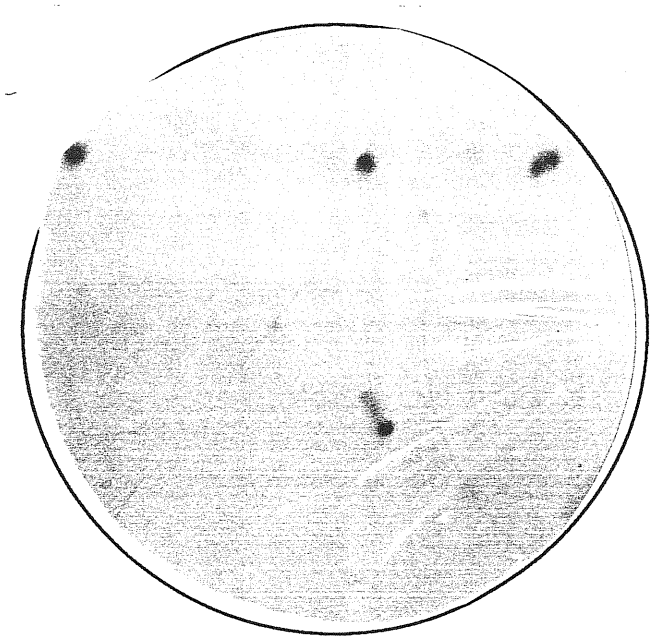


Figure 11: Autoradiography upon hybridization of E.coli recombinant clone no 173

3.2. DNA SEQUENCING AND CHARACTERIZATION

For the sequencing purpose plasmid DNAs was isolated by alkali lysis. After denaturation of the DNA with NaOH, a Sephacryl R S-400 spun column was used to remove both the NaOH and any contaminants which could interfere with sequencing. For dideoxy sequencing with T7 sequencing™ kit (Pharmacia/LKB) universal and reversal primers were used in order to get sequence data from both terminus. The sequencing of clone no. 50 was completed by using additional internal synthetic primers. The nucleotide sequence data are shown in figure 12. These data were subjected to computer analysis and it was observed that the sequence of clone no 50 was 100% homologous to human nucleolin cDNA sequence (from 816 to 1867). The alignment is shown in Fig 13.
The sequence of clone no 173 was done from both the ends only (Fig 14,15). These data were also found to be identical to the nucleolin sequence (Fig 16, 17). The complete nucleotide and corresponding amino-acid sequence of nucleolin is shown in figure 18 and 19.
Clone no 184 was also sequenced from both the ends and computer analysis didn't reveal any homology to the nucleolin or any other sequence in the data bank. Re-screening of original clone no 184 was done and it was found to be false-positive.

3.3. ATPase ACTIVITY

ATPase activity of HDH III and HDH V was measured as reported in Materials and Method. DNA dependent ATPase activity of 100 ng HDH III was present at a level of 18 pmol of ATP hydrolysed in 30 min.
The DNA dependent ATPase activity of 0.3 ng HDH V was present at a level of approximately 40 pmol of ATP hydrolysed in 15 min.

```
   1  ggccgcgggt gctgaagatc ccggagcacg tacacccgaa ggccgccatc
  51  gccgtcgctt ggcttcttct ggactcatct gcgccacttg tccgcttcac
 101  actccgccgc catcatggtg aagctcgcga aggcaggtaa aaatcaaggt
 151  gaccccaaga aaatggctcc tcctccaaag gaggtagaag aagatagtga
 201  agatgaggaa atgtcagaag atgaagaaga tgatagcagt ggagaagagg
 251  tcgtcatacc tcagaagaaa ggcaagaagg ctgctgcaac ctcagcaaag
 301  aaggtggtcg tttccccaac aaaaaaggtt gcagttgcca caccagccaa
 351  gaaagcagct gtcactccag gcaaaaaggc agcagcaaca cctgccaaga
 401  agacagttac accagccaaa gcagttacca cacctggcaa gaagggagcc
 451  acaccaggca aagcattggt agcaactcct ggtaagaagg gtgctgccat
 501  cccagccaag ggggcaaaga atggcaagaa tgccaagaag gaagacagtg
 551  atgaagagga ggatgatgac agtgaggagg atgaggagga tgacgaggac
 601  gaggatgagg atgaagatga aattgaacca gcagcgatga aagcagcagc
 651  tgctgccccc gcctcagagg atgaggacga tgaggatgac gaagatgatg
 701  aggatgacga tgacgatgag gaagatgact ctgaagaaga agctatggag
 751  actacaccag ccaaaggaaa gaaagctgca aaagttgttc ctgtgaaagc
 801  caagaacgtg gctgaggatg aagatgaaga agaggatgat gaggacgagg
 851  atgacgacga cgacgaagat gatgaagatg atgatgatga agatgatgag
 901  gaggaggaag aagaggagga ggaagagcct gtcaaagaag cacctggaaa
 951  acgaaagaag gaaatggcca aacagaaagc agctcctgaa gccaagaaac
1001  agaaagtgga aggcacagaa ccgactacgg ctttcaatct ctttgttgga
1051  aacctaaact ttaacaaatc tgctcctgaa ttaaaaactg gtatcagcga
1101  tgtttttgct aaaaatgatc ttgctgttgt ggatgtcaga attggtatga
1151  ctaggaaatt tggttatgtg gattttgaat ctgctgaaga cctggagaaa
1201  gcgttggaac tcactggttt gaaagtcttt ggcaatgaaa ttaaactaga
1251  gaaaccaaaa ggaaaagaca gtaagaaaga gcgagatgcg agaacacttt
1301  tggctaaaaa tctcccttac aaagtcactc aggatgaatt gaaagaagtg
1351  tttgaagatg ctgcggagat cagattagtc agcaaggatg ggaaaagtaa
1401  agggattgct tatattgaat ttaagacaga agctgatgca gagaaaacct
```

```
   1  ggatgaagat gaagaagagg atgatgagga cgaggatgac gacgacgacg
  51  aagatgatga agatgatgat gatgaagatg atgaggagga ggaagaagag
 101  gaggaggaag agcctgtcaa agaagcacct ggaaaacgaa agaaggaaat
 151  ggccaaacag aaagcagctc ctgaagccaa gaaacagaaa gtggaaggca
 201  cagaaccgac tacggctttc aatctctttg ttggaaacct aaactttaac
 251  aaatctgctc ctgaattaaa aactggtatc agcgatgttt ttgctaaaaa
 301  tgatcttgct gttgtggatg tcagaattgg tatgactagg aaatttggtt
 351  atgtggattt tgaatctgct gaagacctgg agaaagcgtt ggaactcact
 401  ggtttgaaag tctttggcaa tgaaattaaa ctagagaaac caaaaggaaa
 451  agacagtaag aaagagcgag atgcgagaac acttttggct aaaaatctcc
 501  cttacaaagt cactcaggat gaattgaaag aagtgtttga agatgctgcg
 551  gagatcagat tagtcagcaa ggatgggaaa agtaaaggga ttgcttatat
 601  tgaatttaag acagaagctg atgcagagaa aacctttgaa gaaaagcagg
 651  gaacagagat cgatgggcga tctatttccc tgtactatac tggagagaaa
 701  ggtcaaaatc aagactatag aggtggaaag aatagcactt ggagtggtga
 751  atcaaaaact ctggttttaa gcaacctctc ctacagtgca acagaagaaa
 801  ctcttcagga agtatttgag aaagcaactt ttatcaaagt accccagaac
 851  caaaatggca aatctaaagg gtatgcattt atagagtttg cttcattcga
 901  agacgctaaa gaagctttaa attcctgtaa taaaagggaa attgagggca
 951  gagcaatcag gctggagttg caaggaccca ggggatcacc taatgccaga
1001  agccagccat ccaaaactct gtttgtcaaa ggcctgtctg aggataccac
1051  tg
```

Figure 12: Nucleotide sequence of clone no.50

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                                                10        20        30
IV-50R                                          GGATGAAGATGAAGAAGAGGATGATGAGGA
                                                ||||||||||||||||||||||||||||||
nucl.  TGTTCCTGTGAAAGCCAAGAACGTGGCTGAGGATGAAGATGAAGAAGAGGATGATGAGGA
         790       800       810       820       830       840

               40        50        60        70        80        90
IV-50R CGAGGATGACGACGACGACGAAGATGATGAAGATGATGATGATGAAGATGATGAGGAGGA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  CGAGGATGACGACGACGACGAAGATGATGAAGATGATGATGATGAAGATGATGAGGAGGA
         850       860       870       880       890       900

              100       110       120       130       140       150
IV-50R GGAAGAAGAGGAGGAGGAAGAGCCTGTCAAAGAAGCACCTGGAAAACGAAAGAAGGAAAT
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  GGAAGAAGAGGAGGAGGAAGAGCCTGTCAAAGAAGCACCTGGAAAACGAAAGAAGGAAAT
         910       920       930       940       950       960

              160       170       180       190       200       210
IV-50R GGCCAAACAGAAAGCAGCTCCTGAAGCCAAGAAACAGAAAGTGGAAGGCACAGAACCGAC
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  GGCCAAACAGAAAGCAGCTCCTGAAGCCAAGAAACAGAAAGTGGAAGGCACAGAACCGAC
         970       980       990      1000      1010      1020

              220       230       240       250       260       270
IV-50R TACGGCTTTCAATCTCTTTGTTGGAAACCTAAACTTTAACAAATCTGCTCCTGAATTAAA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  TACGGCTTTCAATCTCTTTGTTGGAAACCTAAACTTTAACAAATCTGCTCCTGAATTAAA
        1030      1040      1050      1060      1070      1080

              280       290       300       310       320       330
IV-50R AACTGGTATCAGCGATGTTTTTGCTAAAAATGATCTTGCTGTTGTGGATGTCAGAATTGG
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  AACTGGTATCAGCGATGTTTTTGCTAAAAATGATCTTGCTGTTGTGGATGTCAGAATTGG
        1090      1100      1110      1120      1130      1140

              340       350       360       370       380       390
IV-50R TATGACTAGGAAATTTGGTTATGTGGATTTTGAATCTGCTGAAGACCTGGAGAAAGCGTT
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  TATGACTAGGAAATTTGGTTATGTGGATTTTGAATCTGCTGAAGACCTGGAGAAAGCGTT
        1150      1160      1170      1180      1190      1200

              400       410       420       430       440       450
IV-50R GGAACTCACTGGTTTGAAAGTCTTTGGCAATGAAATTAAACTAGAGAAACCAAAAGGAAA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  GGAACTCACTGGTTTGAAAGTCTTTGGCAATGAAATTAAACTAGAGAAACCAAAAGGAAA
        1210      1220      1230      1240      1250      1260

              460       470       480       490       500       510
IV-50R AGACAGTAAGAAAGAGCGAGATGCGAGAACACTTTTGGCTAAAAATCTCCCTTACAAAGT
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  AGACAGTAAGAAAGAGCGAGATGCGAGAACACTTTTGGCTAAAAATCTCCCTTACAAAGT
        1270      1280      1290      1300      1310      1320

              520       530       540       550       560       570
IV-50R CACTCAGGATGAATTGAAAGAAGTGTTTGAAGATGCTGCGGAGATCAGATTAGTCAGCAA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  CACTCAGGATGAATTGAAAGAAGTGTTTGAAGATGCTGCGGAGATCAGATTAGTCAGCAA
        1330      1340      1350      1360      1370      1380
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                580        590        600        610        620        630
IV-50R GGATGGGAAAAGTAAAGGGATTGCTTATATTGAATTTAAGACAGAAGCTGATGCAGAGAA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  GGATGGGAAAAGTAAAGGGATTGCTTATATTGAATTTAAGACAGAAGCTGATGCAGAGAA
        1390       1400       1410       1420       1430       1440

                640        650        660        670        680        690
IV-50R AACCTTTGAAGAAAAGCAGGGAACAGAGATCGATGGGCGATCTATTTCCCTGTACTATAC
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  AACCTTTGAAGAAAAGCAGGGAACAGAGATCGATGGGCGATCTATTTCCCTGTACTATAC
        1450       1460       1470       1480       1490       1500

                700        710        720        730        740        750
IV-50R TGGAGAGAAAGGTCAAAATCAAGACTATAGAGGTGGAAAGAATAGCACTTGGAGTGGTGA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  TGGAGAGAAAGGTCAAAATCAAGACTATAGAGGTGGAAAGAATAGCACTTGGAGTGGTGA
        1510       1520       1530       1540       1550       1560

                760        770        780        790        800        810
IV-50R ATCAAAAACTCTGGTTTTAAGCAACCTCTCCTACAGTGCAACAGAAGAAACTCTTCAGGA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  ATCAAAAACTCTGGTTTTAAGCAACCTCTCCTACAGTGCAACAGAAGAAACTCTTCAGGA
        1570       1580       1590       1600       1610       1620

                820        830        840        850        860        870
IV-50R AGTATTTGAGAAAGCAACTTTTATCAAAGTACCCCAGAACCAAAATGGCAAATCTAAAGG
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  AGTATTTGAGAAAGCAACTTTTATCAAAGTACCCCAGAACCAAAATGGCAAATCTAAAGG
        1630       1640       1650       1660       1670       1680

                880        890        900        910        920        930
IV-50R GTATGCATTTATAGAGTTTGCTTCATTCGAAGACGCTAAAGAAGCTTTAAATTCCTGTAA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  GTATGCATTTATAGAGTTTGCTTCATTCGAAGACGCTAAAGAAGCTTTAAATTCCTGTAA
        1690       1700       1710       1720       1730       1740

                940        950        960        970        980        990
IV-50R TAAAAGGGAAATTGAGGGCAGAGCAATCAGGCTGGAGTTGCAAGGACCCAGGGGATCACC
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  TAAAAGGGAAATTGAGGGCAGAGCAATCAGGCTGGAGTTGCAAGGACCCAGGGGATCACC
        1750       1760       1770       1780       1790       1800

               1000       1010       1020       1030       1040       1050
IV-50R TAATGCCAGAAGCCAGCCATCCAAAACTCTGTTTGTCAAAGGCCTGTCTGAGGATACCAC
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  TAATGCCAGAAGCCAGCCATCCAAAACTCTGTTTGTCAAAGGCCTGTCTGAGGATACCAC
        1810       1820       1830       1840       1850       1860
```

Figure 13: Sequence alignment of clone no.50 with human nucleolin

```
  1  gtgctcatac ctcagaagaa aggcaagaag gctgctgcaa cctcagcaaa
 51  gaaggtggtc gttttcccca acaaaaaagg ttgcagttgc cacaccagcc
101  aagaaagcag ctgtcactcc aggcaaaaag gcagcagcaa cacctgccaa
151  gaagacagtt acaccagcca aagcagttac cacacctggc aagaagggag
201  ccacaccagg caaagcattg gtagcaactc ctggtaagaa gggtgctgcc
251  atccagccaa ggggcaaaga atgcagagtc agagag
```

Figure 14: Nucleotide sequence of clone no. 173 sequenced with universal primer

```
  1  ggccaggtgc ttctttgaca ggctcttcct cctcctcttc ttcctcctcc
 51  tcatcatctt catcatcatc atcttcatca tcttcgtcgt cgtcgtcatc
101  ctcgtcctca tcatcctctt cttcatcttc atcctcagcc acgttcttgg
151  cttcacagga acaactttgc agcttcttcc ttggctg
```

Figure 15: Nucleotide sequence of clone no.173 sequenced with reversel primer

```
                                                  10         20         30
IV173U                                          GTGCTCATACCTCAGAAGAAAGGCAAGAAG
                                                ||  ||||||||||||||||||||||||||
nucl.  GATGAAGAAGATGATAGCAGTGGAGAAGAGGTCGTCATACCTCAGAAGAAAGGCAAGAAG
     220        230        240        250        260        270

               40         50         60         70         80         90
IV173U GCTGCTGCAACCTCAGCAAAGAAGGTGGTCGTTTTCCCCAACAAAAAAGGTTGCAGTTGC
       |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
nucl.  GCTGCTGCAACCTCAGCAAAGAAGGTGGTCG-TTTCCCCAACAAAAAAGGTTGCAGTTGC
     280        290        300        310         320        330

              100        110        120        130        140        150
IV173U CACACCAGCCAAGAAAGCAGCTGTCACTCCAGGCAAAAAGGCAGCAGCAACACCTGCCAA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  CACACCAGCCAAGAAAGCAGCTGTCACTCCAGGCAAAAAGGCAGCAGCAACACCTGCCAA
      340        350        360        370        380        390

              160        170        180        190        200        210
IV173U GAAGACAGTTACACCAGCCAAAGCAGTTACCACACCTGGCAAGAAGGGAGCCACACCAGG
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
nucl.  GAAGACAGTTACACCAGCCAAAGCAGTTACCACACCTGGCAAGAAGGGAGCCACACCAGG
      400        410        420        430        440        450

              220        230        240        250        260
IV173U CAAAGCATTGGTAGCAACTCCTGGTAAGAAGGGTGCTGCCAT-CCAGCCAA-GGGGCAAA
       |||||||||||||||||||||||||||||||||||||||||| |||||||| ||||||||
nucl.  CAAAGCATTGGTAGCAACTCCTGGTAAGAAGGGTGCTGCCATCCCAGCCAAGGGGGCAAA
      460        470        480        490        500        510

      270        280
IV173U GAATGCAGAGTCAGAGAG
       |||||
nucl.  GAATGGCAAGAATGCCAAGAAGGAAGACAGTGATGAAGAGGAGGATGATGACAGTGAGGA
      520        530        540        550        560        570
```

Figure 16: Sequence alignment of clone no 173 sequenced with universal primer and human nucleolin

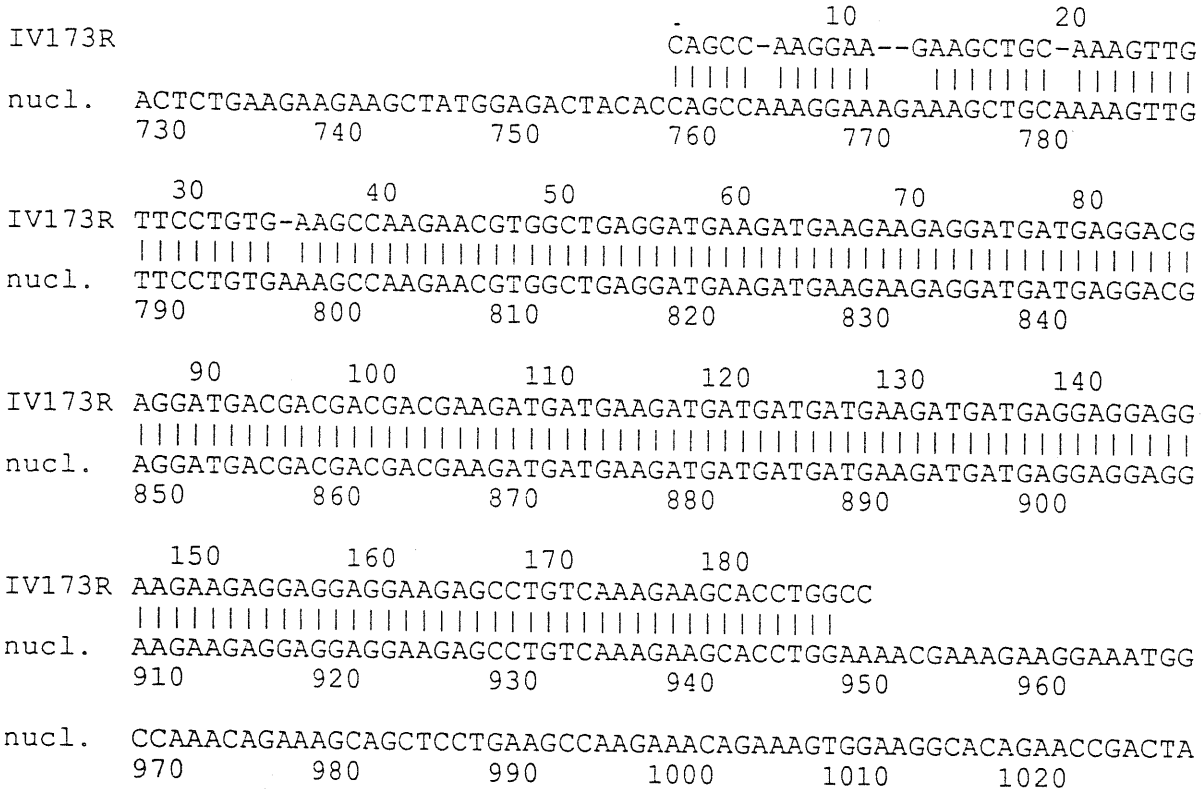

Figure 17. Sequence alignment of clone no. 173 sequenced with reversel primer and human nucleolin

```
   1  ggccgcgggt gctgaagatc ccggagcacg tacacccgaa ggccgccatc
  51  gccgtcgctt ggcttcttct ggactcatct gcgccacttg tccgcttcac
 101  actccgccgc catcatggtg aagctcgcga aggcaggtaa aaatcaaggt
 151  gaccccaaga aaatggctcc tcctccaaag gaggtagaag aagatagtga
 201  agatgaggaa atgtcagaag atgaagaaga tgatagcagt ggagaagagg
 251  tcgtcatacc tcagaagaaa ggcaagaagg ctgctgcaac ctcagcaaag
 301  aaggtggtcg tttccccaac aaaaaaggtt gcagttgcca caccagccaa
 351  gaaagcagct gtcactccag gcaaaaaggc agcagcaaca cctgccaaga
 401  agacagttac accagccaaa gcagttacca cacctggcaa gaagggagcc
 451  acaccaggca aagcattggt agcaactcct ggtaagaagg gtgctgccat
 501  cccagccaag ggggcaaaga atggcaagaa tgccaagaag gaagacagtg
 551  atgaagagga ggatgatgac agtgaggagg atgaggagga tgacgaggac
 601  gaggatgagg atgaagatga aattgaacca gcagcgatga aagcagcagc
 651  tgctgccccc gcctcagagg atgaggacga tgaggatgac gaagatgatg
 701  aggatgacga tgacgatgag gaagatgact ctgaagaaga agctatggag
 751  actacaccag ccaaaggaaa gaaagctgca aaagttgttc ctgtgaaagc
 801  caagaacgtg gctgaggatg aagatgaaga agaggatgat gaggacgagg
 851  atgacgacga cgacgaagat gatgaagatg atgatgatga agatgatgag
 901  gaggaggaag aagaggagga ggaagagcct gtcaaagaag cacctggaaa
 951  acgaaagaag gaaatggcca aacagaaagc agctcctgaa gccaagaaac
1001  agaaagtgga aggcacagaa ccgactacgg ctttcaatct ctttgttgga
1051  aacctaaact ttaacaaatc tgctcctgaa ttaaaaactg gtatcagcga
1101  tgtttttgct aaaaatgatc ttgctgttgt ggatgtcaga attggtatga
1151  ctaggaaatt tggttatgtg gattttgaat ctgctgaaga cctggagaaa
1201  gcgttggaac tcactggttt gaaagtcttt ggcaatgaaa ttaaactaga
1251  gaaaccaaaa ggaaaagaca gtaagaaaga gcgagatgcg agaacacttt
1301  tggctaaaaa tctcccttac aaagtcactc aggatgaatt gaaagaagtg
1351  tttgaagatg ctgcggagat cagattagtc agcaaggatg ggaaaagtaa
1401  agggattgct tatattgaat ttaagacaga agctgatgca gagaaaacct
```

1451 ttgaagaaaa gcagggaaca gagatcgatg ggcgatctat ttccctgtac

1501 tatactggag agaaaggtca aaatcaagac tatagaggtg gaaagaatag

1551 cacttggagt ggtgaatcaa aaactctggt tttaagcaac ctctcctaca

1601 gtgcaacaga agaaactctt caggaagtat ttgagaaagc aacttttatc

1651 aaagtacccc agaaccaaaa tggcaaatct aaagggtatg catttataga

1701 gtttgcttca ttcgaagacg ctaaagaagc tttaaattcc tgtaataaaa

1751 gggaaattga gggcagagca atcaggctgg agttgcaagg acccagggga

1801 tcacctaatg ccagaagcca gccatccaaa actctgtttg tcaaaggcct

1851 gtctgaggat accactgaag agacattaaa ggagtcattt gacggctccg

1901 ttcgggcaag gatagttact gaccgggaaa ctgggtcctc caaagggttt

1951 ggttttgtag acttcaacag tgaggaggat gccaaggagg ccatggaaga

2001 cggtgaaatt gatggaaata aagttacctt ggactgggcc aaacctaagg

2051 gtgaaggtgg cttcgggggt cgtggtggag gcagaggcgg ctttggagga

2101 cgaggtggtg gtagaggagg ccgaggagga tttggtggca gaggccgggg

2151 aggctttgga gggcgaggag gcttccgagg aggcagagga ggaggaggtg

2201 accacaagcc acaaggaaag aagacgaagt ttgaatagct tctgtccctc

2251 tgctttccct tttccatttg aaagaaagga ctctggggtt tttactgtta

2301 cctgatcaat gacagagcct tctgaggaca ttccaagaca gtatacagtc

2351 ctgtggtctc cttggaaatc cgtctagtta acatttcaag ggcaataccg

2401 tgttggtttt gactggatat tcatataaac tttttaaaga gttgagtgat

2451 agagctaacc cttatctgta agttttgaat ttatattgtt tcatcccatg

2501 tacaaaacca ttttttccta caaaaaaaaa aaaaaaaaaa aaaaaaaaaa

2551 aaaaaaaaaa aaaaaaaaaa a

Figure 18: Nucleotide sequence of human nucleolin

MVKLAKAGKNQGDPKKMAPPPKEVEEDSEDEEMSEDEEDDSSGEEVVIPQKKGKKAAATSAKKVVVSPTK

KVAVATPAKKAAVTPGKKAAATPAKKTVTPAKAVTTPGKKGATPGKALVATPGKKGAAIPAKGAKNGKNA

KKEDSDEEEDDDSEEDEEDDEDEDEDEDEIEPAAMKAAAAAPASEDEDDEDDEDDEDDDDEEDDSEEEA

METTPAKGKKAAKVVPVKAKNVAEDEDEEEDDEDEDDDDDEDDEDDDDEDDEEEEEEEEEEPVKEAPGKR

KKEMAKQKAAPEAKKQKVEGTEPTTAFNLFVGNLNFNKSAPELKTGISDVFAKNDLAVVDVRIGMTRKFG

YVDFESAEDLEKALELTGLKVFGNEIKLEKPKGKDSKKERDARTLLAKNLPYKVTQDELKEVFEDAAEIR

LVSKDGKSKGIAYIEFKTEADAEKTFEEKQGTEIDGRSISLYYTGEKGQNQDYRGGKNSTWSGESKTLVL

SNLSYSATEETLQEVFEKATFIKVPQNQNGKSKGYAFIEFASFEDAKEALNSCNKREIEGRAIRLELQGP

RGSPNARSQPSKTLFVKGLSEDTTEETLKESFDGSVRARIVTDRETGSSKGFGFVDFNSEEDAKEAMEDG

EIDGNKVTLDWAKPKGEGGFGGRGGGRGGFGGRGGGRGGRGGFGGRGRGGFGGRGGFRGGRGGGGDHKPQ

GKKTKFE

Figure 19: Amino-acid sequence of human nucleolin

DISCUSSION

HELICASES- IMPORTANT ENZYME IN MANY DNA METABOLIC PROCESSES

Whereas for most of the DNA helicases isolated from E.coli a reasonable attribution of function in DNA metabolism has been obtained, in eukaryotes this objective has not yet been obtained with clear-cut evidence for anyone of the different enzymes already isolated. Even in a relatively simple prokaryote , such as S.cerevisiae, no helicase essential for DNA replication process has been unambiguously recognized, and only in the case of radiation-deficient strains (the Rad 3 gene) a function in excision repair has been recognized for a helicase moving in 5'-3' direction (45).
The situation is certainly more difficult for the helicases isolated from plants, Xenopus or mammalian organs and cells; over seventeen such enzymes have been purified so far, but their function has still eluded the investigators and no obvious similarity among the different mammalian helicases has been observed, such as could allow a classification analogous to the one which was successfully made for the different eukaryotic DNA polymerases (46). Among animal virus-encoded DNA helicases, the T-antigen associated helicase activity stands out insofar that its role in DNA replication initiation and fork advancement is well proven (47,48,49). On the other hand, the variety of viral strategies evolved to reproduce inside the infected cells are such that one cannot expect any obvious similarity between the molecules involved in similar processes on the viral and mammalian chromosomes. Similarly from the properties of prokaryotic helicases, whose function in different aspects of DNA metabolism has been well established, no reasonable inference is possible as regards the properties of comparable enzymes in eukaryotes.

Tuteja and coworkers (33,34,35,36) have initiated a systematic study of DNA helicases in Hela cells. Four of them have been

already purified and their properties including polarity of translocation and reaction requirement described.
Isolation and characterization of helicase genes and their cloning into expression vector would be an important step in further DNA helicase characterization and their role in DNA metabolism.
We started the isolation of HDH IV gene using polyclonal antibody previously purified by protein A Sepharose Before use for screening antibody was adsorbed with E.coli antigen.
Antibody screening revealed three positive clones no. 50,173 and 184 which were found to have insert of 1 kb, 0.7 kb and 1kb respectively.
The sequencing clone no. 50 demonstrated 100% similarity to human nucleolin. The amino acid microsequencing of HDH IV amino terminus also indicated some similarity of the helicase with amino terminus of human nucleolin. HDH IV only contained one extra KMA sequence. Having these findings in mind we continued to screen the library to find a full lenght clone.
Two additional clones no. 173 and 184 were also sequenced. Out of those clone 184 appeared to be false positive.
Sequence of the ends of clone no.173 also revealed homology with nucleolin. These data led us to believe that HDH IV is human nucleolin. Figure 20 shows the position of two sequenced clones within the known nucleolin sequence.

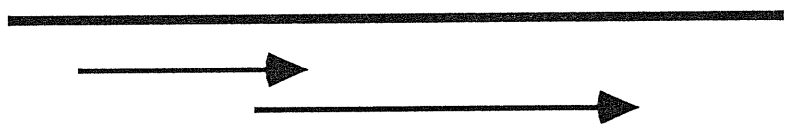

Figure 20:Position of two clones within nucleolin sequence

Tuteja et al. (50) have also shown that HDH IV is phosphorylated by cdc2 kinase. It has been demonstrated previously that nucleolin is phosphorylated by cdc2 kinase (66)
The following observation:
a) Molecular weight of HDH IV and human nucleolin is 100 kD

b) Amino-terminus of HDH IV and human nucleolin are almost identical
c) Both are substrates for cdc2 kinase
d) The nucleotide sequence of HDH IV and human nucleolin are homologous

lead to the conclusion that HDH IV is most probably identical to human nucleolin.
Further characterization of in vitro expressed HDH IV and its role in DNA metabolism are in progress.

NUCLEOLIN

Nucleolin is though to be a multifunctional protein involved in the control of early step of synthesis and /or processing of ribosomal RNA (51) It was also reported that it binds spacer region between the repeated rRNA genes (52), and thus may organize nucleolar chromatin providing the specificity for transcription by RNA polymerase I which seems to be species-specific. Central part of the protein which has alternating hydrophilic and hydrophobic segment binds RNA and has same similarity to the proteins of spliceosomes. The binding of the nucleolin with early transcribed RNA in the nucleolus together with the properties stated above implicated its role in RNA processing.
What would be the role of nucleolin in those processes as DNA helicase?
Nucleolin is located in the fibrillar centers deep in the nucleoli which contain as well the genes encoding rRNA, Pol I, topoisomerase I and probably some transcription factors. Those factors are probably complexed with non transcribing genes but held in an inactivated state. It may be possible that nucleolin by covalent modification may switch on the transcription. The phosphorylation of nucleolin by Casein kinase II has been demonstrated in many tissues (53,54,55,56) The phosphorylation occurs on serine residues and the activities of Casein Kinase and rRNA synthesis were found to be dependent on cell growth of Hela cells (57), on hormonal stimuli in hepatocytes (57) and lymphosarcoma P1798 (58). N-terminal phosphorylated portion of nucleolin is known to be highly susceptible to proteolysis (59). In

Chinese-hamster cell, the synthesis of pre-rRNA is inhibited by the protease inhibitor leupeptin which ihibits the nucleolin degradation probably important for rDNA transcription (60) The phosphorylation of nucleolin in 3T3-F442A cells enhanced its proteolytic degradation to 30 kD and 72 kD peptides (61). All this facts raises the possibility that nucleolin phosphorylation and its preferential degradation may be something to do with pre-rRNA synthesis and maturation of rRNA. Schneider et al. showed that the major degradation product of nucleolin, p60 could be dephosphorylated by two phosphatases: P1 and P2A (62). Both are closely associated with the chromatin but phosphatase P1 inhibited by histone H1. It is likely that histone may act as a regulator of nuclear dephosphorylation. Upon receiving some external signal which leads to phosphorylation of nucleolin by casein kinase II, the nucleolin can bind to histone H1 leading to decondensation of chromatin. In this way phosphatase P1 can now becomes activated and, after proteolytic cleavage of nucleolin dephosphorylate the brakdown product p60. It is important to stress that those phosphatase do not dephosphorylate nucleolin.When human fibroblast were treated with the inhibitor of those phosphatase, okadaic acid, dephosphorylation was consequently inhibited leading to disturbance of ribosomal biosynthesis and blocking of cell growth

FKBPs are proteins which can directly bind to immunosuppressant drugs FK506 and rapamycin. They shares two conserved domains: a drug binding site and a peptidyl prolyl cis-trans isomerase activity (63).

One member of this family FKBP25 is localized to the nucleus and it can associate and be phosphorylated by casein kinase II. Transport of FKBP25 to the nucleus in enhance by casein kinase II phosphorylation and this could be the way how its nuclear localization could be regulated. Interestingly a nucleolin has been demonstrated to be a part of the complex as well as being the major protein phosphorylated (64).

FKBP25 may as a peptidyl-prolyl isomerase function as molecular chaperones (65) which facilate protein folding, subunit assemblea, maybe important for ribosomal protein assembly with rRNA, and organelle compartmentalization. Both CK II and nucleolin may be a substrate for FKBP25.

If HDH IV is indeed nucleolin its helicase activity might be activated by casein kinase II phosphorylation contributing to initiation of transcription by melting dsDNA which thus becomes available to Pol I.
It would be interesting to demonstrate if HDH IV is also a substrate for casein kinase II and if different phosphorylation state of HDH IV changed the kinetics of its helicase activity. The direct protein-protein interaction of HDH IV and Pol I can not also be excluded.
Nucleolin is also phosphorylated on threonine residues by mitosis specific kinase $p34^{cdc2}$ which could be important in the mitotic reorganization of the nucleolus (66). There are nine TPXK motifs-phosphorylation sites for cdc2 kinase present in the basic region which might be important for association with spacer region. Phosphorylation might weaken its association with spacer leading to mitotic reorganization of the nucleolus. During mitosis chromosome condensation is indeed accompanied by the disaggregation of the nucleolus, as well as switch-off of rRNA synthesis.
Tuteja et al have demonstrated that HDH IV is also a substrate for cdc2 kinase (50)

NUCLEOLIN SHUTTLE BETWEEN NUCLEUS AND CYTOPLASM

Borer et al. demonstrated that nucleolin, previously being detected into a nucleus can shuttle constantly between nucleus and cytoplasm (67). They were monitoring the equilibration of nucleolin between nuclei present in interspecies heterokaryons, and on observing the antigen mediated nuclear accumulation of cytoplasmaticly injected antibodies upon binding with cytoplasmatic nucleolin which contain karyophilic sequence. Nucleolin thus could be involved in the transport of ribosomal proteins being translated in a cytoplasm and probably this would be energy dependent process because the appearance of antigen-antibody complex in the nucleus is temperature.dependent.
The protein may be also involved in the transport of preribosomal particles.

The finding of cytoplasmic localization of nucleolin raises the possibility that the phosphorylation might also occur in the cytoplasm by some cytoplasmatic kinases regulating its complex formation with ribosomal proteins. Phosphorylation might also be a mechanism for signal transfer, informing the nucleus for the cytoplasmatic demands for ribosomal production. One of the candidate might be MAP kinase since it site of phosphorylation is similar to cdc2.

The finding of the nucleolin cytoplasmatic localization could explained the presence of HDH IV in a cytosol which we were detected by western blot (data non shown). Further purification of the HDH IV from the cytosol and characterization of its helicase and ATPase activity might be interesting since those activities may be significantly modulated or even absent.

The studies to find the role of HDH IV in DNA/RNA metabolism are in progress.

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