Journal of
Cell Science &
Molecular Biology

Open Science Publications

Volume 5, Issue 1 - 2023

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Heat Shock Induced Triple Helix DNA and it's Refolding to Duplex by HSP70 in Yeast

Research Article

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Article Information: Submission: 10/12/2022; Accepted: 11/01/2023; Published: 16/01/2023

Abstract

Heat can damage and inhibit the synthesis and repair of damage to DNA, proteins, RNA. Heat shock increases DNA damage by crosslinking and breakages. Crosslinking creates the multi strand DNA, which down regulates many genes. Triple helix is a major form of multi strand DNA reported to exist. Using poly purine templates for BLAST, number of possible triple helical structures were predicted. Frame shift assays using denaturing PAGE, UV spectra, melting complexes, DMSO foot printing and RNAse T treatment were analyzed for triple helix formation. Heat shock caused accumulation of triple helix DNA in yeast. Heat shock proteins were reported and found to bind the triple helical DNA. Double stranded DNA structure was retrieved from triple helix with the help of HSP 70. This study confirms the possible role of HSP 70 for refolding of misfolded DNA like that of proteins.

Keywords: Triple helix DNA; Heat shock; HSP; Thermotolerance; Yeast

Introduction

Research over years has yielded clear evidence that DNA is the molecule responsible for the inheritance of traits from one generation to the next. DNA is usually double stranded; two strands of nucleotides are attached to one another via Hydrogen bonds between bases. Many of these sequences adopt the orthodox right-handed B form, probably for the majority of the time, with Watson-Crick A:T and G:C bp. However, at least 10 non-B conformations are formed, perhaps transiently, at specific sequence motifs as a function of negative supercoil density, generated in part by transcription, protein binding, and other factors.

Multistranded DNA structures have attracted a great attention in recent years, as they possibly play a substantial role in chromosomal DNA organization and regulating gene expression [1-4]. Triple helix DNA was first discovered in a complex of poly (A) and poly (U) by Felsenfeld, Davis and Rich in 1957 [5]. Arnott and coworkers established the triple helix DNA model by X-ray diffraction analyses in 1974 and ever since researchers are resolving DNA triple helix structures [6,7]. Studies of triple helix DNA have been paid much more attention because of its importance as a tool for DNA sequencing, gene control and therapeutic applications [1]. The triple helix provides means to design powerful artificial endonuclease when the third strand is coupled with a cleaving agent. The triple helix forming activity also holds strong promise in the areas of genome mapping. Triple helix-DNA provide potential tools for altering gene function by either repressing transcription, inhibiting DNA replication or inducing site-specific mutagenesis and recombination [8,9]. It was found that the population of triple helix-forming target sequences is much more abundant, especially in promoter zones, which suggests a tremendous potentiality for triple helix strategy in the control of gene expression [10]. Studies from human genetics

ISSN: 2350-0190

and from model organisms indicate that the Triple DNA itself plays a major role in its own mutability [11]. The intermolecular triple helix has been shown to inhibit gene expression in vivo, including a case demonstrating the inhibition of HIV-1 transcription in infected human cells. Medically related issues have been vigorous motivators of triple-stranded research. At least 20 hereditary neurological diseases are caused by the expansion of simple triple helix sequences in either coding or non-coding regions [12]. It has been previously demonstrated that triple helix formation at a promoter can block the binding of various transcription factors thereby inhibiting transcription. Another mechanism may be to prevent transcriptional elongation. Intermolecular DNA triple helixes have pronounced sequence recognition properties and have been used successfully to decrease transcription from specific genes [13].

Cells and tissues are challenged constantly by exposure to extreme conditions that cause acute and chronic stress. Consequently, survival has necessitated the evolution of stress response networks to detect, monitor, and respond to environmental changes [14]. Prolonged exposure to stress interferes with efficient operations of the cell, with negative consequences on the biochemical properties of proteins, Nucleic acids that, under ideal conditions, exist in thermodynamically stable states. In vitro studies revealed the formation of triple helix DNA by heat shock [15]. The ethidium bromide fluorescence assay detects DNA interstrand crosslinks following heat denaturation of DNA [16]. Heat shock protein's role in refolding of thermal stress induced misfolded proteins is well established, where as few reports stating the similar role of HSPs on Nucleic acid aggregates [17].

There are now several areas of research that target the problem of triple helix stability. One possible strategy, with which we concerned, is to use compounds that bind spherically to triple helix (not duplex) DNA, thereby facilitating the formation of double helix structures. In present study we were attempted to found heat induced triple helix formation and its reversal by HSP70 treatment.

Materials & Methods

Culture Conditions

Microorganism: *Saccharomyces cerevisiae - VS*$_3$ was isolated from soil samples collected from hot regions near the Kothagudem Thermal Power Plant located in Khammam District, TS, India. The organism was isolated, mutated by UV and identified as *Saccharomyces cerevisiae -VS*$_3$ strain in our lab [18]. It was maintained on Yeast extract Peptone Dextrose agar medium (YEPD) (1% Yeast Extract, 2% Peptone, 2% Glucose, 2% Agar-Agar, p^H 6.0).

Chemicals & enzymes

Human HSP 70, Yeast Lyticase, RNAse was obtained from Sigma Aldrich.

Remaining chemicals and media reagents are obtained from HIMEDIA chemicals.

Prediction of Triple helix DNA in Yeast

Query sequence GAGAAAAATGAA with poly purines, reported to form triple helix DNA was used to BLAST (Search for short, nearly exact matches) against yeast genome database available with NCBI Gene bank [19]. Results obtained (top 10) were checked for their function analysis from NCBI database [20].

Confirmation of Triple helix DNA

Yeast Chromosome preparation and Heat shock

Thermotolorant yeast *Saccharomyces cerevisiae* VS$_3$ strain was grown in YEPD broth for 48 hours at 30°C and 150 rpm. Yeast cells washed with 0.5M PBS and were suspended in protoplast solution (0.5M PBS, 6M sorbitol, 2mM Beta Mercapto ethanol , 100U lyticase and 1μl of Rnase (50 ng/μl)). The mixture was incubated at 30°C and 50 rpm for 2 hours. Samples were collected at an interval of 10 minutes up to 2 hours and checked for protoplast conversion by methylene blue staining, spherical shape of protoplasts and hypotonic rupture of protoplasts. After 90% cells were converted to protoplasts, heat shock was given at 50 and 60°C for 45 minutes.

Nondenaturing PAGE assay for triple helix formation

Rnase and Lyticase treated yeast suspension was centrifuged at 22000 rpm and supernatant containing enzymes were removed. The pellet was used for phenol extraction of DNA. Then 5ml of the mixture was combined with 1 ml of 6X aqueous glycerol containing xylenecyanole and Bromphenol Blue. The products were resolved in 20×20 cm slabs of 4% gel (acrylamide/bisacrylamide 19:1 with Ethidium Bromide) in TAE at 24°C for 16-18 h at 60 mA, 110 V. Bands were visualized by Transilluminator in gel documentation.

Dimethyl Sulfate (DMS) Footprinting

Heat shocked chromosome preparations (250μl) was purified by phenol extraction and treated with 10 μl of 1:40 dilution of DMS in dimethyl sulfoxide for 3 min at 0°C. Reactions were stopped by DNA precipitation with ethanol. For both DNA cleavage of DMS-treated substrates and removal of crosslink, pellets were dried and resuspended in 100 μl of 0.1 M NaOH and heated to 90°C for 1 h. After an additional precipitation, samples were resuspended in 5 μl of loading buffer and loaded on an 8 M urea/4% poly acrylamide gel and used for electrophoresis under denaturing conditions.

HSP Treatment

After heat shock at 50°C protoplasts were treated with pure HSP70 (Sigma) and incubated at 37°C for 1 hour. Samples were extracted with phenol/chloroform, and products were precipitated with ethanol. Theses samples were used for electrophoresis and estimation of T_m.

Thermal stability

Thermal stability of complexes was assessed by monitoring the UV absorption of the solution in Spectronic spectrophotometer while the temperature of DNA sample was steadily raised at 1°C/min from 25 to90°C. The concentration of DNA was 100 μg/10μl in 10mM Tris HCL buffer.

RNase Hydrolysis

Heat shocked and DMS printed chromosomal DNA (2ml) were separately treated with 5 μl of RNase solution (50 ng/μl) in a final volume of 50 μl at 37°C for 1 h. Reaction mixtures were extracted with phenol/chloroform, and products were precipitated with ethanol.

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Results & Discussion

The existence of triple helix sequences has been studied by using bioinformatics tools [10], hence we also approached bioinformatics mode of predicting triples sequences in yeast genome.

In BLAST analysis, many of the genes (80%) forming triple helix DNA were not yet characterized. Remaining are expressed but down regulated after the heat shock [21].

Prediction of triple helix-forming sequences in Yeast (Table 1)

Query sequence GAGAAAAATGAA**,** Blasted against *S.cerevisiae* database.

Confirmation of Triple helix DNA in heat shocked yeast

Microscopic examination showed spherical Protoplasts. At 25th minute, 80% protoplast formation was noted. When these protoplasts were diluted in distilled water, decrease in protoplast count was noted due to hypotonic rupture. Protoplasts were stained by methylene blue and observed for loss of membrane bound oxidases (blue stained). Protoplasts were also stained by using geimsa stain for chromosomes. Chromosomal DNA isolated from protoplasts showed 16 separate bands indicating each chromosome as a separate band. Protoplasts (>80%) after heat shock were found to aggregate the chromosomal DNA. After heat shock, number of bands was decreased with increase in shock temperature, indicating the formation of complexes.

Dimethyl sulfate (DMS) methylates G residues, creating an adduct that can be broken chemically. Triple helix complexes are formed and treated with DMS and then the reaction is stopped with ethanol. Subsequent treatment with hot alkali breaks the DNA backbone at methylated sites [22]. Triple helix DNA complexes were found to be protected from DMS mediated DNA cleavage due to hindered G in major rove [23]. Heat shocked (50^{0}C) and DMS foot printed yeast showed almost similar aggregates like heat-shocked yeast. Rnase T cleaves single strand RNA/DNA [24]. Rnase T treatment to heat shocked yeast DNA yielded many bands confirming triple helical DNA formation by heat shock (Figure 1).

Melting Complexes

Melting temperature of un shocked yeast DNA was 55^{0}C, whereas in heat shocked yeast DNA, it was 61^{0}C. Increased T_m (melting temperature) of heat shocked yeast Genomic DNA also confirms the formation of triple helix DNA. Triple helix binding proteins were detected in human and yeast for their role in making linear duplexes enabling proper chromosome segregation [25,26]. The molecular chaperones are housekeeping molecules that assist in the folding and prevention of the aggregation of proteins and nucleic acids, as well as participating in the elimination of ubiquitinated molecules [**17**]. Heat shocked DNA was treated with Heat shock protein (HSP 70) for 20 minutes. After Hsp, treatment DNA was phenol extracted and used for detection of melting temperatures. T_m (melting temperature) of Heat shocked and Hsp treated DNA is similar to un shocked yeast. Heat shocked and HSP treated Genomic DNA was found to have the T_m 54^{0}C. Heat shocked yeast chromosomes were reversed by Hsp 70 treatment (Figure 2).

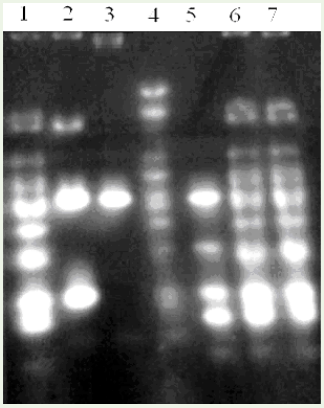

Figure 1: 1) Un shocked Yeast chromosomes; 2) Heat shocked Yeast chromosomes (at 50^{0}C), 3) Heat shocked Yeast chromosomes (at 60^{0}C), 4) Heat shocked and RNAse treated Yeast Chromosomes, 5) Heat shocked and DMS treated Yeast chromosomes, 6) Heat shocked and Hsp treated Yeast chromosomes, 7) Un shocked Yeast chromosomes.

Table 1: Probable Triple helix DNA forming Genes obtained by BLAST.

S.No	Gene Sequence name	Chromosome	Score	E value	Function
1	gi|1015838|emb|Z49618.1|SCYJR118C	X	24	3.0	Unknown
2	gi|476045|emb|X78993.1|SCRACII70Kd	II	24	3.0	Given below
	1) Proliferating cell nuclear antigen, 2) Mitochondrial regulator of splicing, 3) Constitutive acid phosphotase, 4)Repressible acid phosphotase 5) Probable protein kinase, 6) Alpha mannosyl transferase, 7) Unknown Protein, 8) Glucose repression mediator protein, 9) UV damage repair protein, 10) Alpha-aminoadipate reductase, 11) transketolase, 12) Elongation factor EF-1-alpha, 13) Small nuclear ribonucleoprotein, 14) Nuclear factor for cytB synthesis, 15) Probable transfer RNA-Gly synthetase, 16) Ribosomal protein YmL36 fragment, 17) Transcription factor TFIIIC, 18) Unknown Protein 19) Regulator of carbon catabolite repression				
3	gi|1360372|emb|Z73215.1|SCYLR043C	X	24	3.0	Unknown
4	gi|1360370|emb|Z73214.1|SCYLR042C	XII	24	3.0	Unknown
5	gi|4086|emb|X69881.1|SCORF12	Unidentified DNA	24	3.0	Unknown
6	gi|1301875|emb|Z71310.1|SCYNL034W	XIV	24	3.0	Unknown
7	gi|536461|emb|Z35966.1|SCYBR097W	II	24	3.0	Unknown
8	gi|1420165|emb|Z74949.1|SCYOR041C	XV	24	3.0	Unknown
9	gi|6318278|gb|U14000.1|SCU14000	Mms4P	24	3.0	Unknown
10	gi|1079672|gb|U39205.1|SCU39205	XVI	24	3.0	Given below
	1) Bts1p: Geranylgeranyl diphosphate synthase 2) Ald6p: Acetaldehyde dehydrogenase 3) Pdr12p: Multidrug resistance transporter				

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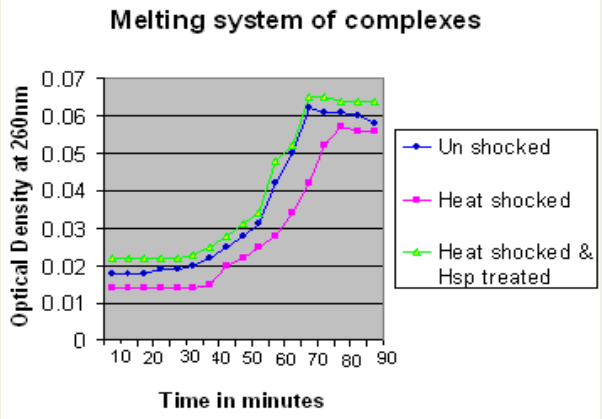

Figure 2: Melting curves of yeast chromosome complexes. Tm of un shocked yeast Chromosomal DNA: 55^{0}C, Tm of Heat shocked yeast Chromosomal DNA: 61^{0}C, Tm of Heat shocked and Hsp treated yeast Chromosomal DNA: 54^{0}C.

After heat shock, the formation of Triple helix DNA confirms *invitro* reports (19). These triple helix structures may be responsible for blockage of genes after heat shock. Triple helix DNA was converted to double helix by the treatment of HSP 70. HSP70 can be used to convert inactive multi strand structures of DNA into the active double helix and subsequent *invitro* gene expression to characterize the gene.

Triple helix DNA was known to create the barrier for DNA polymerase [1]. Down regulation of several genes after hear shock can be correlated with triple helix formation, over expression of genes can be explained by their activation by HSPs or selection of different polymerase or its component like *Bacillus* during sporulation [27]. *Saccharomyces cerevisiae* - VS_3 produces all major types of HSPs along withHSP70 after heat stress. HSP70 along with others HSPs converting triple helix DNA structures to duplexes to retain activity of most of genes and subsequently conferring in thermotolerance.

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