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hnRNP A2/B1 binds specifically to single stranded vertebrate telomeric repeat $TTAGGG_n$

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ABSTRACT

We have previously isolated a protein from mouse liver nuclei that specifically binds to single stranded $(TTAGGG)_n$ repeats. TTAGGG is the telomeric repeats of mammals and we therefore named the new protein single stranded telomere binding protein (sTBP). Further studies now identify sTBP as heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 on the basis of amino acid sequence determination and antibody reactivity. A2 and B1 form a major part of the protein component of hnRNP particles and are abundant nuclear proteins. Unexpectedly, A2/B1 has a high specificity for binding to the RNA equivalent of TTAGGG, UUAGGG, but under the same conditions does not appear to have a strong affinity for a number of other RNA species.

INTRODUCTION

The focus of this project was to identify proteins that were involved in binding to telomere repeats, $(TTAGGG)_n$. These TTAGGG repeats are highly conserved and are found in all vertebrates (1). They are the only element shown to be necessary for replication of the ends of eukaryotic chromosomes (2). In Oxytricha and Euplotes it has been shown that the ends of chromosomes are protected from ligation and exonucleolytic attack by a protein complex that binds to the protruding DNA strand containing G-rich repeats (3,4). In species where analysis has been possible this strand protrudes 12–16 nucleotides beyond the complementary C-strand (5,6). DNA binding proteins with strong sequence specificity for telomeric terminal repeats could have a role in telomere replication and hence chromosome stability.

This paper reports the results of amino acid sequence determination on sTBP, the protein we previously isolated as having single stranded $(TTAGGG)_n$ binding activity (7). This allowed the identification of sTBP as hnRNP A2/B1. Surprisingly, this protein binds not only to a single strand of $(TTAGGG)_n$ but also to the RNA equivalent $(UUAGGG)_n$ in a very sequence specific manner. This raises the possibility that this protein may have more than one function and could have a role to play in the biology of telomeres.

MATERIALS AND METHODS

Peptide cleavage and sequencing

100–150μg of sTBP was concentrated to a volume of 50μl by centrifugation in a centricon 10 miniconcentrator (Amicon) following manufacturer's instructions. The concentrated protein was mixed with 350μl 70% formic acid. A small crystal of cyanogen bromide was added and the reaction incubated for 16 hours at 25°C in darkness. 400μl of H_20 was added and the reaction taken to dryness in a Savant Speedvac concentrator SVC100H. The final purification of the peptides and sequencing was carried out by the WELMET protein characterisation facility, University of Edinburgh. The dried pellet was dissolved in acetonitrile and separated on an Applied Biosystems 130A Microbore HPLC. Separated peptides were analysed by an Applied Biosystems 427A Protein Microsequencer.

Gel electrophoresis and immunoblotting

Mouse liver nuclear proteins were resolved on 15% SDS/PAGE gels and electro-blotted onto nitro-cellulose membrane as previously reported (7). 6mm strips were cut from appropriate regions of the blot and blocked in 10% horse serum, Tris buffered saline pH 8.2 (TBS), Tween 20 (0.05%) for 15–30 minutes at room temperature. The filters were incubated with antibody (see below) for 16 hours at room temperature and then washed twice in TBS, Tween 20 (0.05%). The immunoblot was then incubated in Vectastain ABC anti mouse secondary antibody for 30 minutes, washed twice in TBS, Tween 20 (0.05%), incubated in Vectastain ABC reagent for 30 minutes, washed twice in TBS, Tween 20 (0.05%) and finally washed in 50mM Tris pH7.0. Sites of antigen-antibody binding were detected by incubating with 0.5μg/ml 4-chloro-1-napthol, 50mM Tris pH7.0, 0.01% hydrogen peroxide.

hnRNP monoclonal antibody 7A9

The monoclonal antibody (Mab) was a gift from G.Dreyfuss at the Howard Hughes Medical Institute Research Laboratories (8). It was prepared by removing culture media from 7A9 hybridomas, filtering through a 0.2μm membrane and adding sodium azide to 0.02%.

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Immunoprecipitation

1ml of hybridoma culture supernatant from either anti hnRNP Mab 7A9 or anti Wilms tumour protein (WT1) Mab 48.83.23 (both IgG 2a subclass) was mixed with 80ml of sheep anti-mouse IgG magnetic beads (DYNAL) for 2 hours at room temperature. The beads were then washed four times in 1ml binding buffer,(10mM Tris−HCl, pH7.5, 1mM EDTA, 4% glycerol, 0.1% Triton-X100, 10mM β-mercaptoethanol) and finally re suspended in 100μl binding buffer.

Gel retardation binding reactions (as described below) using ^{32}P labelled oligos 785 and 717 (both detailed in table 1.) with and without sTBP and including 1ug of sonicated E.coli DNA were mixed in a total volume of 25ml and incubated for 10 minutes at room temperature.

Oligonucleotide 785 with sTBP and without sTBP and oligonucleotide 717 with sTBP and without sTBP binding reactions were mixed with either 20ul of bead bound Mab 7a9 or Mab 8.3. The amount of radioactivity in each reaction was measured by Cerenkov counting in a Packard liquid scintillation analyser. Binding reactions and bead bound Mab were then mixed for 2 hours at room temperature. The reactions were then washed 3 times in binding buffer, and the amount of radioactivity present in the solid phase was measured.

DNA and RNA

Plasmid DNA was prepared by standard methods. Plasmid pBSTTA contains TTAGGG repeats from pHutel-2-end (9). $(UUAGGG)_n$ RNA was transcribed in vitro from linear pBSTTA with T7 RNA polymerase (promega). Oligonucleotides were synthesised on an Applied Biosystems 381A synthesiser. Total RNA was extracted from mouse brain using guanidinium thiocyanate (10).

Gel retardation

Binding reactions were carried out in 10mM Tris−HCl, pH7.5, 1mM EDTA, 4% glycerol, 0.1% Triton-X100, 10mM β-mercaptoethanol. 0.5ng ^{32}P labelled oligonucleotide GGGTT-A$(GGGTTA)_5$GGG was mixed with various competitor nucleic acids and incubated with 0.6μg sTBP at room temperature for 10 minutes in a total of 20μl. Samples were separated on a 5% polyacrlyamide gel (electrophoresis conditions, 0.5×TBE buffer for 100 min at 10V/cm at room temperature). The gel was dried at 80°C under vacuum for 45 min onto 3MM paper then exposed to Kodak X-AR autoradiographic film. The developed autoradiograph was aligned with the dried gel to visualise the areas of bound and unbound activity which were then excised. Activity from each area was measured by Cerenkov counting in a Packard liquid scintillation analyser.

RESULTS

Sequence analysis

sTBP (purified as described in reference 7) was chemically cleaved with cyanogen bromide (CnBr) and sequenced as detailed in Materials and Methods. The CnBr cleavage reaction produced three major peaks when analysed on a microbore HPLC. The largest peak was blocked at the amino terminus and therefore could not be sequenced. Peaks 2 and 3 were sequenced to give 3 peptides of 14, 15 and 32 amino acids. Computer searches of the Swissprot database using BLASTA showed that all three peptides had perfect homology to hnRNP A2 and hnRNP B1

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1    MEKTLETVPL ERKKREKEQF RKLFIGGLSF ETTEESLRNY YEQWGKLTDC

51   VVMRDPASKR SRGFGFVTFS SMAEVDAAMA ARPHSIDGRV VEPKRAVARE

101  ESGKPGAHVT VKKLFVGGIK EDTEEHHLRD YFEEYGKIDT IEIITDRQSG

151  KKRGFGFVTF DDHDPVDKIV LQKYHTINGH NAEVRKALSR QEMQEVQSSR

201  SGRGGNFGFG DSRGGGGNFG PGPGSNFRGG SDGYGSGRGF GDGYNGYGGG

251  PGGGNFGGSP GYGGGRGGYG GGGPGYGNQG GGYGGGYDNY GGGNYGSGNY

301  NDFGNYNQQP SNYGPMKSGN FGGSRNMGGP YGGGNYGPGG SGGSGGYGGR

351  SRY
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Figure 1. Amino acid sequence of heterogeneous nuclear ribonucleoproteins A2/B1. The 12 amino acids unique to B1 are underlined with a double line. Sequences derived from the 3 sTBP peptides are underlined with a single line.

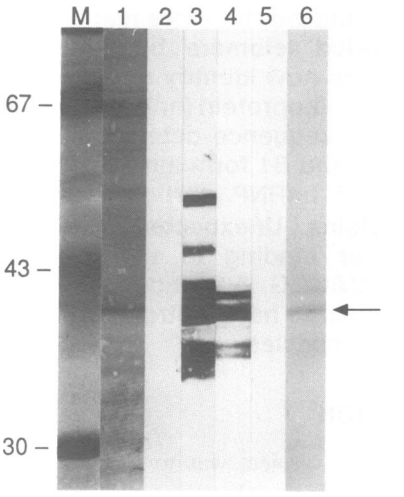

Figure 2. Immunoblot of 0.2M mouse liver salt wash (lanes 1−3, approximately 22μg per lane) and sTBP (lanes 4−6, approximately 3μg per lane). Lane 1, Coomassie blue stained. Lane 2, incubated with the second antibody only. Lane 3, incubated with Mab 7A9 . Lane 4, incubated with 7A9. Lane 5, incubated with the second antibody only. Lane 6, sTBP coomassie blue stained. Lane M, molecular weight markers of 67,43 and 30kDa. The arrow indicates the position of sTBP.

(Figure1). Discrimination between hnRNPs A2 and B1 was not possible on the basis of the sequence determined because these regions are identical in the two proteins.

Immunoblotting

When affinity purified sTBP was resolved on a SDS/PAGE gel other faint protein bands in addition to the major 36kDa component were always present (Fig 2,lane 4). To confirm the identity of the major band with hnRNP A2/B1 we probed a western blot containing purified sTBP and 0.2M mouse liver salt wash with monoclonal antibody (Mab) 7A9 which reacts with hnRNPs A, B, G, E, H and L. In Figure 2 it is clear that the major band (approximately 36kDa) is detected most strongly by the Mab. The presence of other immunoreacting bands suggests

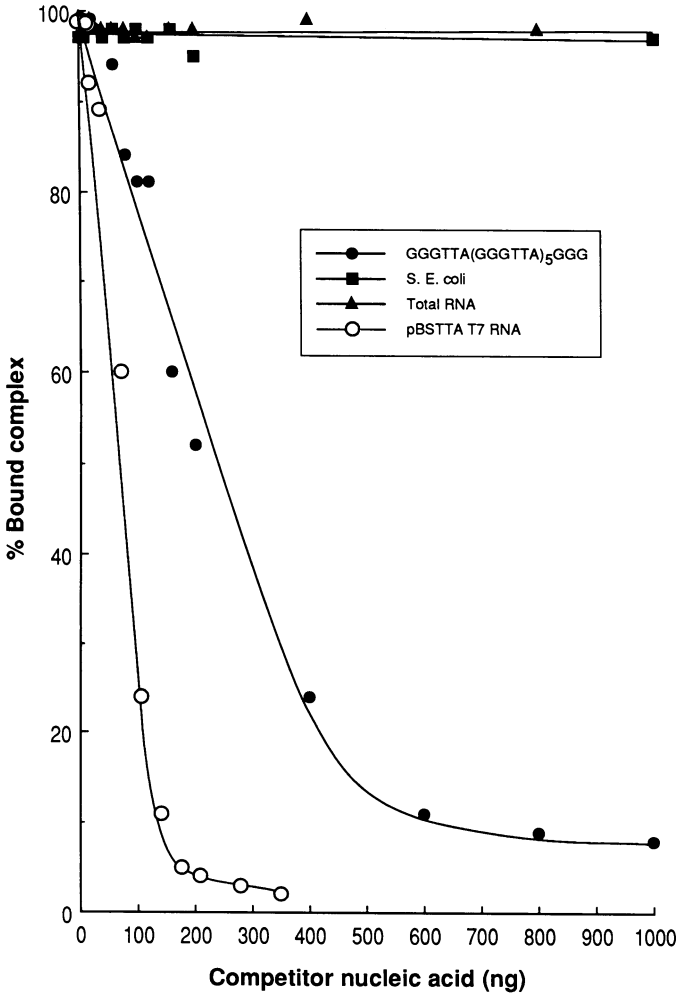

Figure 3. Sequence specificity of sTBP gel retardation activity. Gel retardation was carried out as described in material and methods using 0.5ng ^{32}p labelled GGGTTA(GGGTTA)$_5$GGG with competitor nucleic acid (oligonucleotide GGGTAA(GGGTTA)$_5$GGG represented by a closed circle, sonicated E.coli represented by a closed square, total RNA represented by a closed triangle and pBSTTA T7 RNA represented by a open circle).

that other but not all hnRNPs co-purify with sTBP and therefore also have some specific affinity for (TTAGGG)$_n$ repeats.

Mab 7A9 was also used in immunofluorescence experiments to investigate the biological role of the DNA binding activity of hnRNP A2/B1. Fixed and non-fixed metaphase chromosome spreads were probed with the antibody. No apparent localisation of signal on the chromosomes could be detected whereas interphase nuclei present amongst the metaphase chromosomes were intensely and uniformly stained (data not shown).

Immunoprecipitation

To demonstrate whether hnRNP A2/B1 was the protein that bound to single stranded TTAGGG$_n$ repeats rather than a co-purifying protein/proteins we attempted to immunoprecipitate the sTBP/TTAGGG$_n$ complex with anti hnRNP Mab 7a9 as described in material and methods.

As shown in table 1, 58% of oligonucleotide 785 bound with sTBP could be immunoprecipitated with Mab 7a9. Only

Table 1. Immunoprecipitation of sTBP/oligonucleotide 785 complex

Reaction	% cpm in immunoprecipitation complex
(785 + sTBP) + Mab 7a9	58.0%
(785 − sTBP) + Mab 7a9	1.5%
(717 + sTBP) + Mab 7a9	3.3%
(717 − sTBP) + Mab 7a9	2.3%
(785 + sTBP) + Mab 84	2.3%
(785 − sTBP) + Mab 84	3.4%
(717 + sTBP) + Mab 84	2.1%
(717 − sTBP) + Mab 84	1.0%

Immunoprecipitation was carried out as described in materials and methods. The complexes formed by the components interaction enclosed in brackets were completed before mixing with the indicated Mab. Oligonucleotide 785−GGGTTA(GGGTTA)5GGG, Oligonucleotide 717-CTCTCCCTTCTCG-AATCGTAACCGTTCGATCGAGAATCGCTGTCCTCTCCAT, Mab 7a9−anti hnRNP, Mab 84−anti Wilms tumour protein (W.T.1), sTBP single stranded telomere binding protein.

background binding (<4%) occurred when sTBP was omitted from the reaction or when an oligonucleotide (717) of a similar size to oligonucleotide 785 was substituted. Furthermore an unrelated Mab (Mab 84) of the same sub-class (IgG 2a) was unable to immunoprecipitate the sTBP/(TTAGGG)$_n$ complex (table 1.) This demonstrates that the complex was binding specifically to the Mab 7a9 binding site and not non-specifically to the magnetic beads/Mab complex.

Gel retardation

Since our results identified sTBP as hnRNP A2/B1 we expected RNA to compete strongly against the formation of the protein/DNA complex. To test this, we used total RNA from mouse brain and (UUAGGG)$_n$ RNA transcribed from a plasmid DNA template as described in Materials and Methods.As shown in Figure 3 both total RNA and sonicated E.coli DNA are ineffective competitors. In contrast GGGTTA(GGGTTA)$_5$GGG and UUAGGG$_n$ compete strongly for the complex. The greater effectiveness of UUAGGG over TTAGGG$_6$ as a competitor may be accounted for by the difference in size of the molecule (500 compared to 36 nucleotides) or may reflect the ability of the protein to bind to RNA. Other T7 RNA polymerase generated transcripts of random 300−400 base pair plasmid inserts were used as competitors in gel retardation assays and were also ineffective (data not shown).

DISCUSSION

The sequence of sTBP demonstrates that it is most likely to be an hnRNP as the 3 peptides sequenced have perfect homology to hnRNP A2/B1. The N-terminal 15 amino acids of sTBP could not be sequenced because of N-terminal modification and in the absence of this sequence it was not possible to distinguish between A2 and B1, because the 12 amino acid insert unique to B1 is found in this region. Immunoblotting with an anti hnRNP monoclonal antibody showed that hnRNP A2/B1 was co-purifying with other hnRNPs during the sTBP purification. There remains the remote possibility that the sTBP activity is a different protein from the hnRNP which binds to the bead bound oligonucleotide and that the amino acid sequence is not derived from this protein. To demonstrate that the binding activity is an hnRNP we have shown that sTBP/telomere oligonucleotide

complex can be specifically immunoprecipitated by an anti hnRNP antibody.

It has been suggested that A2 may be one of the first proteins to bind RNA (11) in hnRNP particle assembly. Planck et al. (12) reported that A2 and B1 are present in a 3:1 ratio in proliferating cells, although this ratio may vary in other stages of cell growth. This could account for the difference in abundance found between tissues for sTBP (7). The 2 strongest bands present in the sTBP immunblot (Figure 2) have a molecular weight of approximately 36 and 38kDa corresponding to A2 and B1. A2 appears to be present in a greater ratio than 3:1. It is unclear whether this is because the A2/B1 ratio is greater in mouse liver nuclei or because A2 has a greater affinity for $(TTAGGG)_6$ than B1.

While it has been extensively shown that hnRNPs are responsible for packaging heterogeneous RNA into spliceosomes (13,14,15,16), the sequence specificity exhibited by A2/B1 suggests that it could also be involved in forming a telomere/protein complex. However we were unable to detect a telomeric location for the protein on metaphase chromosomes. The amount of hnRNP A2/B1 required for this proposed function compared to the nuclear abundance required for its role as an RNA transport protein is negligible and if the protein was present at telomeres the amount might be below our detection limit. HnRNP A1 has previously been reported to have one property which suggests that a dual function of these proteins is possible. It can enhance nucleic acid reassociation rates *in vitro* by up to 3000 fold at physiological temperatures (17), which in terms of telomeres might have two possible roles. Firstly such an activity might serve to keep the telomeres in double stranded form and secondly it might increase the rate at which the RNA template in a telomerase RNP can find its genomic counterpart for telomere elongation to initiate.

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