ASE-1: a novel protein of the fibrillar centres of the nucleolus and nucleolus organizer region of mitotic chromosomes

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Abstract. A novel nucleolar component has been identified and cloned using a human autoimmune serum. This antigen, as inferred from the cDNA sequence, is an Mr 55000 protein. Immuno blot analysis, however, of both the native protein and the in vitro translation products of the cDNA showed that they migrate on SDS-PAGE at an apparent molecular mass of 90000 A BLAST search using the cDNA sequence indicated that it is in an anti-sense orientation to and overlaps the gene of the DNA repair enzyme ERCC-1. An open reading frame, without a translational start site, had been observed by others in this region of the chromosome 19 (19q13.3) and the putative protein was termed ASE-1 (Anti-Sense to ERCC-1). Our cDNA is a full-length equivalent of that open reading frame. ASE-1 was found to contain two domains that are present in a number of nucleolar specific proteins originating from a variety of organisms: a glycine-, arginine- and phenylalanine-rich putative nucleotide interaction domain and an alternating basic/acidic region. Indirect immunofluorescence analysis using antibodies generated to cloned regions of ASE-1 indicated that this protein occurs at the fibrillar centres of the nucleolus in interphase, the putative sites of rDNA transcription, and during cell division it is localized to the nucleolar organizer regions of the chromosomes. ASE-1 co-localises with the RNA polymerase I transcription initiation factor UBF/NOR-90 throughout all stages of the cell cycle and these two proteins associate with each other in vitro.

Introduction

The nucleolus is a complex nuclear component that displays both morphological and functional compartmentalization. Three distinct regions have been identified: the fibrillar centres (FC), surrounded by the dense fibrillar components (DFCs), which are in turn encompassed by the granular component (GC). The FCs are through to be the sites of transcription of rDNA by virtue of the localization of RNA polymerase I, topoisomerase I and the class I transcription factor NOR-90/UBF to this region (Muller et al. 1985, Reimer et al. 1987; Thiry 1993; Fritzler et al. 1995). The surrounding DFC is known to contain the unprocessed nascent rRNA transcripts, mature 28S and 18S rRNA (Scheer and Weisenberger 1994). Partially assembled ribosomal particles of 15–20 nm can be visualized in the GC (Melese and Xue 1995).

The extensive nature of this list and the recent discovery of No55, indicate that human autoimmune sera...
still have the potential to identify additional nucleolar components. We have screened a human cDNA library with a human autoimmune serum reactive with the nucleolus and have identified and cloned a cDNA that codes for a novel Mr 55 000 protein. This autoantigen is localized to the FCs of the nucleolus during interphase and the NORs of the chromosomes during cell division. This cDNA encompasses a previously identified open reading frame, ASE-1 (Van Duin et al. 1989; Martin-Gallardo et al. 1992), which is positioned in an antisense orientation to and overlaps the gene for the DNA excision repair enzyme ERCC-1 (Van Duin et al. 1986).

Materials and methods

Human serum information. The two human sera used in this study (11980, ASE-1 and J0, NOR-90/UBF) were obtained from the serum bank of the Advanced Diagnostic Laboratory at The University of Calgary. Sera were stored at −20 C or −80 C until required for serological studies.

Cell culture and indirect immunofluorescence. The specificity of autoantibodies for ASE-1 antigens was first detected by indirect immunofluorescence (IIF) using monolayer cultures of HeLa cells (American Type Tissue Collection, Rockville Md.). The cells were grown on coverslips and fixed for 10 min in 3% paraformaldehyde in Dulbecco’s phosphate-buffered saline (D-PBS) and permeablized in 0.5% Triton X-100 in D-PBS. In some experiments, actinomycin D (1 µg/ml) (Sigma, St. Louis Mo.) was added to the cultures for 4 h prior to processing. Fixed preparations were briefly washed with D-PBS and incubated for 1 h at 37 C with the appropriate serum. Following three washes in D-PBS, the samples were incubated for 1 h at 37 C in a fluorescein- or C3-conjugated anti-human, rabbit or mouse IgG(H+L) (Dako, Santa Barbara, Calif.). After incubation, the specimens were washed in D-PBS, counterstained with 4’,6-diamidino-2-phenyl-indole, mounted in 90% glycerol containing P-phenylenediamine and observed using a Nikon Optiphot fluorescence microscope. Images were recorded on Ilford HP-5 film.

Double-label IIF experiments were performed as described above except that two separate incubations of the desired primary and secondary antibodies were used. Digitally computed optical sections were collected as previously described (Rattner et al. 1996).

Isolation of cDNA clones. The human autoimmune serum, designated 11980, or an appropriately immunized rabbit serum was diluted 1:2000 and used to screen a HeLa uni-ZAP XR cDNA library (Stratagene, La Jolla, Calif.). Approximately 1 x 106 recombinant phage were plated on lawns of Escherichia coli BB4 and incubated at 37 C for 4 h. Expression of fusion proteins was induced by overlaying with nitrocellulose filters impregnated with isopropylthiogalactoside (IPTG, Gibco BRL, Burlington, ON) for 4 h. Reactive plaques were detected by processing the filters with an immunoblotting protocol (see below). Positive clones were plaque purified and in vivo excised into pBluescript plasmid using R408 helper phage as described in the manufacturer’s instructions (Stratagene, La Jolla, Calif.). The nucleotide sequences of the isolated clones were determined using the dsDNA Cycle Sequencing System (Gibco BRL, Burlington, ON). Nucleic acid and protein sequences were analysed by the University of Wisconsin Genetics Computer Users Group Sequence Analysis Software package Version 8.1 for UNIX computers (Devereux et al. 1984). Comparisons with known sequences were performed by BLAST (Altschul et al. 1990) on the NCBI Internet Server. The complete 5’ sequence of the cDNA was determined by 5’ RACE (rapid amplification of cDNA ends) as previously described (Whitehead et al. 1996). The 3’ end of the cDNA was also determined by RACE using essentially the same protocol except that the dT anchor primer was used instead of oligo(dT) to prime the first-strand cDNA. The appropriate cDNA sequence-specific primers were designed from the published sequence corresponding to the open reading frame (ORF) of ASE-1 (Van Duin et al. 1989; Martin-Gallardo et al. 1992).

Generation of fusion proteins and antibody production. All fusion proteins were produced as glutathione S-transferase (GST) fusions from the pGEX family of vectors (Pharmacia, Baie d’Urfe, QB). The first fusion protein (FP-1), encompassing amino acids (aa) 20 to 146, was generated by digestion of the 5’ RACE product with BstUI and Stul and inserting the resulting DNA fragment into the Smal site of pGEX 5x-3. The 5’ overlap of the Ncol and EcoRI fragment (corresponding to aa 180 to 363) from clone IBH-23 were filled in with the Klenow fragment of E. coli DNA polymerase I. The resulting fragment was subcloned into the Smal site of pGEX 5x-1 to produce FP-2. The cDNA insert from clone IBH-3 was digested with Ncol and the resulting 5’ overlap was filled in which Klenow fragment and then further digested with EcoRI. The resulting fragment was subcloned into the EcoRI and Smal sites of pGEX 5x-3 to produce FP-3 (corresponding to aa 363 to 488). Colonies found to contain inserts that were isolated and the insert orientation confirmed by DNA sequencing. Selected clones were grown in 2YT medium to an OD600 of 0.6–0.8 and induced to produce GST fusion proteins by the addition of IPTG to a final concentration of 0.1 mM. Inductions were performed for 2 h at 37 C. The fusion proteins were purified according to the manufacturer’s protocol (Pharmacia, Baie d’Urfe, QB), suspended in PBS and used to immunize mice and/or rabbits by subcutaneous injection.

The peptide EAPTGRDKKRKQQQ, corresponding to aa 494 to 507, was chosen for chemically synthesis (Peptide Synthesis Core Facility, The University of Calgary) owing to its predicted antigenicity. This peptide was coupled to keyhole limpet haemocyanin (KLH) as hapten, suspended in PBS and used to immunize rabbits by subcutaneous injection. The antibody generated to this peptide was designated anti-IBH-KLH.

Immunoblotting analysis. Protein samples were resuspended in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled for 5 min, separated by 10% SDS-PAGE and then electroblotted to nitrocellulose. The nitrocellulose was blocked by incubation in TBS-Tween (25 mM TRIS-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 3% skin milk for 30 min. Strips cut from the filters were then overlayed with the appropriate sera at a dilution of 1:1000 for 1 h. Unbound antibody was removed by washing the strips three times with TBS-Tween for 10 min each. Secondary antibody (horseradish peroxidase-conjugated polyvalent goat anti-human or mouse or rabbit IgG, A, M, 1:2000, (Zymed Laboratories, South San Francisco, Calif.) was incubated with the filters for 30 min. Filters were washed three times with TBS-Tween for 10 min each. Immuno-reactive bands were visualized by enhanced chemiluminescence (ECL) according to the manufacturer’s protocol (Amersham, Arlington Heights, Ill.).

In vitro transcription and translation. As no cDNA clones were isolated that contained the amino-terminus of ASE-1, a clone was prepared by fusing amino-terminus (PCR) polymerase chain reaction (PCR) products with a carboxyl-terminus clone (IBH-23). PCR amplification of a first-strand HeLa cDNA was performed using the sense primer ACCGGCTCGAGATGGAGGAGCAGCAGGC containing a XhoI site near the 5’ end and covering the sequence for all amino acids coded for by the first exon and the codon for the first amino acid encoded in the second exon) and the antisense primer CTTCTTGGTGATCCTCCGGAG (corresponding to bases 1854 to 1875, see Fig. 2a). This PCR product was digested with XhoI and Ncol and cloned into the XhoI/Ncol sites of pBSKNSco (this vector had an Ncol site introduced into the EcoRI site of pBSKs), the resulting plasmid being called pBsxN. The 733 bp Ncol/XbaI fragment isolated from IBH-23 was then subcloned into the Ncol and XbaI sites of pBsxN, resulting in a clone that contained the entire ASE-1 coding region. Plasmid DNA containing the entire ASE-1