Immunodetection of the Ribosomal Transcription Factor UBF at the Nucleolus Organizer Regions of Fish Cells

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ABSTRACT. A human autoimmune serum to nucleolus organizer regions (NORs) has been used to localize these structures at the light microscopic level in carp and trout tissue culture cells. In interphase cells, the immunofluorescence pattern indicates that the NORs autoantigens are contained exclusively within the nucleolus of carp epithelial (EPC) and trout gonad (RTG) cells. This fluorescence is punctate rather than uniform, and presumably represents transcriptional complexes of ribosomal DNA. During mitosis, the autoantigens are detected by immunofluorescence microscopy at the chromosomal nucleolus organizer regions of condensed chromosomes, indicating that a considerable quantity of the molecule(s) remains bound to the ribosomal RNA genes. The major nucleolar autoantigen, defined in mammals as the upstream ribosomal binding factor (UBF), has been identified on immunoblots as a 90 kDa protein in extracts from fish cell lines and tissues. Thus, NORs appear to function as nucleation centers for ribosomal RNA together with a complex set of well-conserved protein factors, such as UBF. Our results suggest evolutionary conservation from fish to mammals with respect to ribosomal RNA biosynthesis driven by RNA polymerase I.

NORs are the initiation sites of ribosomal RNA (rRNA) synthesis for nucleologenesis. The number of NORs varies between species and in culture cell lines. Several non-histone proteins are present at NORs in mammalian cells (14, 15). These include RNA polymerase I, DNA topoisomerase I, fibrillarin, nucleolin, and upstream binding factor (UBF). Scleroderma patients make autoantibodies to these nuclear antigens, many of which appear to be well conserved during evolution.

Although the function of the nucleolus was established more than 30 years ago, some fundamental questions remain unsolved; for example the complete set of molecules involved in transcription within the nucleolus and the mechanism of pre-rRNA processing have still to be defined (24, 27). One way of approaching these important problems is to examine the nucleolar structure and its biochemical components in a variety of organisms. Most species of fish have a large number of comparatively small chromosomes (9, 10). However the contribution of karyology to fish taxonomy and systematics, has been minimal. Specific differences in the phenotypes of nucleolus organizer regions (NORs) occur between fish species, and interspecific NOR differences are taxonomically and systematically informative (1, 10, 11). In this study, we have used a human anti-NOR serum to localized NORs by immunofluorescence (IF) in dividing cells and tissues of fish origin.

MATERIALS AND METHODS

Antibodies. Human autoimmune serum directed against NORs was obtained from a patient suffering from rheumatoid arthritis. This antibody, described previously (22), was used without any purification at 1/300 (vol/vol) dilution in 0.1 M phosphate-buffered saline (PBS) for both IF and blotting studies.

Cell lines and IF microscopy. Established cell lines from trout gonad (RTG) and carp epithelium (EPC) were grown on coverslips at 20°C and 28°C, respectively, until near confluence; they were washed briefly in PBS, fixed at room temperature in 3% formaldehyde for 20 min, and permeabilized by incubation with 0.1% Triton X-100 for 2 min. The specimens were incubated for 45 min at 37°C with human anti-NOR serum, washed in PBS for 15 min, followed by incubation at 37°C for 45 min with fluorescein isothiocyanate-conjugated goat anti-human IgG diluted 1/80 (vol/vol) in PBS. Nuclei were stained for DNA with Hoechst 33342 after the antibody treatments (30). Finally, coverslips were mounted in PBS/glycerol 1/9 (vol/vol) on slides and observed under a Zeiss Axioskop fluorescence microscope equipped for epi-illumination.
using appropriate filter combinations.

**ImmunobLOTS.** To detect the ribosomal transcription factor UBF in fish cell extracts, samples were prepared as follows. Whole protein extract from EPC cells was prepared by sonication in buffer A (100 mM Na2HPO4/NaH2PO4 pH 8.0, 150 mM NaCl, 1 mM phenylmethane sulfonylfluoride (PMSF), 1 mM dithiobisreitol, 1 μM EC-64, 2.5 μM DCC, 0.5 μg/ml trypsin inhibitor, 0.5 μg/ml aprotinin) followed by boiling at 100°C for 10 min. This extract was stored at −80°C until further use.

Different tissues from Sparus aurata (spleen, brain, gonad, and kidney) were homogenized in buffer A at 4°C, centrifuged, and soluble proteins were used directly for Western analysis. A control protein extract from HeLa cells was obtained from nuclei isolated in the presence of 0.2% Triton X-100 followed by treatment with buffer A.

Protein samples were separated by discontinuous 10% SDS polyacrylamide gel electrophoresis (PAGE) (16) and transferred to nitrocellulose paper as described (28). Following transfer, the protein-binding sites on the membrane were blocked by a 1 h incubation in PBS containing 5% non-fat dry milk. This was followed by incubation of the nitrocellulose membrane for 16 h in human anti-NOR serum diluted in PBS. Filters were then washed extensively in PBS containing 0.05% Tween 20. In order to visualize the immune complexes, the membrane was incubated for 2 h in PBS containing peroxidase-conjugated goat antihuman IgG diluted 1:2000 in PBS and was further treated with a chloronaphthol solution for band visualization (28, 30). Molecular weight markers and negative controls for the specificity of the antibody reaction (see figure legends) were run in narrow slots alongside experimental samples.

**RESULTS**

**IF staining of fish cell lines with human anti-NOR autoantiserum.** A human autoimmune serum recognizing NORs in mammalian cells was used in indirect IF studies on trout and carp cell lines. By light microscopy, it was evident that this antibody specifically and intensely interacted with the nucleoli of interphase of RTG and EPC cells in culture (Fig. 1). The staining was absent from the cytoplasm and other regions of the nuclei, and revealed several small distinct fluorescence entities within the nucleoli (Figs. 1c, d, 2f). This localization identified sites of rRNA transcription in the nucleoli as shown by treatment of cultured cells with actinomycin D, which resulted in a redistribution of the fluorescent intranucleolar structures (data not shown) into cap-like aggregates at the nucleolar periphery (7, 25). During mitosis, only strong fluorescing spots present on some of the chromosomes were seen following IF staining with the anti-NOR serum (Fig. 2g–j). This pattern was evident from prometaphase to telophase, and was most notable at metaphase. In favorable metaphase plates, such as that shown in Fig. 2i, separate spots on each relevant chromatid could be resolved. For EPC cells, at least four double pairs of stained dots were seen, indicating a minimum of two pairs of chromosomes carrying NORs.

In order to identify the fish antigen recognized by the NOR antibody, the human serum was tested on blots with fish cell extracts of different origins. In RTG and EPC protein extracts, a major 90-kDa polypeptide was identified by immunoblotting (Fig. 3A, lanes 2, 3). This protein showed a similar electrophoretic mobility to that of human UBF proteins (90–92 kDa) (3, 6, 7). Other minor bands were also detected on the blots but their identification as nucleolus antigens require further experimentation. Appropriate controls demonstrated the specificity of this immunoblotting procedure (Fig. 3B). To substantiate this biochemical characterization further, we performed immunoblots with protein extracts from different tissues of the teleost fish gilthead seabream (Sparus aurata) (Fig. 4A). Again, a major antigen of 90 kDa was detected with the human anti-NOR serum in cell extracts taken from the spleen (Fig. 4A lane 1), kidney (lane 2), gonad (lane 3), and liver (lane 4).

**DISCUSSION**

Chromosomal NORs represent sites of the tandemly arranged 18S and 28S rRNA genes (4, 5, 8, 27), intimately involved with protein synthesis (12, 13). In fish species, chromosomal NORs are informative with respect to systematics. Thus, interspecies NOR differences include (i) the number of NOR-bearing chromosomes per genome, (ii) the chromosomal position(s) of the NORs, and (iii) the type(s) of chromosomes upon which the NORs are located.

We have studied the NOR region in several fish cell lines and tissues by IF and immunoblots. For these experiments, we have used a human autoimmune serum that recognizes NORs in a wide range of mammalian cell lines; specifically, it recognizes the ribosomal transcription factor UBF (6, 22). In electron microscopic preparations of sectioned mammalian culture cells, most of the transcription factor UBF is associated with the dense fibrillar material of the nucleoli (22, 23). The antibody has proved to be useful for staining NOR in fish cells. Our observations by IF have shown that the serum specifically stains the nucleolus of interphase fish cells. In addition, our IF study also shows that the rRNA coding sequences are concentrated in clusters of several distinct nucleolar entities. Previous observations of an actinomycin D-induced loss of fluorescence provides support for this conclusion (22, 24, 29). Our IF analysis of the NOR autoantigen localization suggests that UBF is on transcriptionally active regions of the nu-
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Fig. 1. Immunofluorescence staining of fish interphase cells with human autoantibody to NORs. Note the multiple fluorescence speckles in nuclei of EPC cells (c) and RTG cells (d). The same cells were stained for DNA with Hoechst 33342 (a, b). Bar 10 μm.

Of particular interest is the observation that UBF molecules remain associated with the NORs during mitosis. This was initially suggested by biochemical data indicating that UBF polypeptides were among the proteins recovered from isolated metaphase chromosomes (22). Our IF localization studies show that these molecules are not randomly distributed on the mitotic chromosomes but remain in close association with the rDNA genes. Because there is no evidence for rRNA synthesis during metaphase, the UBF bound to the NOR on mitotic chromosomes must be in a state of arrested transcription. The mechanism that prevents UBF actin during mitosis thus provides an interesting model for studying the control of gene activity in fish cells.

It is known that NOR proteins are highly conserved (12, 14). In mammals there are two isoforms of UBF, namely UBF1 and UBF2. The predicted size of the two forms differs by 4.5 kDa (21). In fish cells, we have detected by Western blots one major band of 90 kDa. This band probably represents the product of a single gene in fish, in contrast to the situation in mammals where two genes are involved or where alternative splicing takes place. The strong sequence conservation of UBF in rat, human, and xenopus (3, 19, 21), and now in fish is clear, and it is not surprising that these transcription factors are interchangeable with respect to in vitro transcription assays and in DNAse I footprinting experiments (3, 26). The availability of an anti-NOR serum that recognizes fish UBF, should now make feasible the
Fig. 2. Immunolocalization of NOR proteins during different stages of EPC cell cycle. Staining was detected on mitotic chromosomes as pairs of dots during early prophase (g), prometaphase (h), metaphase (i), and late anaphase (j). The number of mitotic fluorescent spots was constant from cell to cell. Interphase cells are also shown (a, f). Chromosomes were stained for DNA with Hoechst 33342 (b, c, d, e). Bar 10 μm.
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Fig. 4. Immunoblotting analysis of protein extracts from Sparus aurata tissues. (A) Immunoreactivity of cell extracts from spleen (lane 1), kidney (lane 2), gonad (lane 3), and liver (lane 4) to human autoantibody against NORs. A major polypeptide of 90 kDa is detected in all tissues (arrowhead). A human non-immune serum was used as a control in B. Molecular weight standard proteins (lane S) correspond to those in Fig. 3.

isolation of the gene for this transcription factor from expression libraries.

The utility of using chromosomal NORs as taxono-

Fig. 3. Immunoblotting analysis of fish cell extracts with anti-NOR serum (A). A major band was observed in RTG (lane 2) and EPC (lane 3) cells. This band shows similar electrophoretic mobility (arrowhead) to the human autoantigen UBF detected in a HeLa cell extract (lane 1). A human non-immune serum was used as a control in B. Molecular weight standard proteins (lane S) correspond to 200 kDa, 116 kDa, 84 kDa, 58 kDa, 48 kDa, 36 kDa, and 26 kDa (left).

mic and systematic characters has been demonstrated for example, in the North American Cyprinidae (2, 10). Similarly, the localization of NORs in fish species should aid the taxonomic identification of morphologically similar forms. The anti-NOR serum used in this study has proved to be a powerful tool for unequivocally staining NOR chromosomes in fish cells by IF, and for identifying the nucleolar antigen by Western blot. This probe will be used for further biochemical analysis and evolution studies in fish species.

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