Human Autoantibody to RNA Polymerase I Transcription Factor hUBF. Molecular Identity of Nucleolus Organizer Region Autoantigen NOR-90 and Ribosomal RNA Transcription Upstream Binding Factor

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Summary

In dividing eukaryotic cells, nucleoli disperse before mitosis and reform in daughter cells at sites of ribosomal RNA (rRNA) gene clusters that are at the secondary constrictions of chromosomes, called nucleolus organizer regions (NORs). In this study, cDNA clones for a NOR autoantigen (NOR-90) were selected using a specific human autoantibody probe and were subsequently identified to encode an alternative form of the reported human upstream binding factor (hUBF). Results from immunoprecipitation showed that anti-NOR-90 antibodies recognized both forms of hUBF/NOR-90. Our data therefore showed that UBF, a critical factor in the regulation of rRNA transcription, was tightly bound to NOR during mitosis even when rRNA synthesis was thought to be minimal. Furthermore, we identified a nucleolar transcription factor as a novel target for human autoimmune response.

Nucleolus organizer regions (NORs)[1] are the initiation sites for nucleologenesis where prenucleolar bodies converge and fuse to form interphasic nucleoli (1, 2). rRNA synthesis is integrally linked to active NORs (3). The number of NORs varies among species and in cultured cell lines. There is a single NOR located on the X chromosome of the PtK2 cell of rat kangaroos, while there are five NORs located on the acrocentric arms of chromosomes 13, 14, 15, 21, and 22 in humans. Several nonhistone proteins are present at NORs. These include RNA polymerase I, the enzyme essential for rRNA synthesis (4, 5), DNA topoisomerase I, which is important in transcription and DNA replication (6), and the nucleolar U3-RNP protein fibrillarin (2), recently shown to be required for pre-rRNA processing (7, 8). In 1987, using autoantibodies from several patients with the systemic autoimmune disease scleroderma, Rodriguez-Sanchez et al. (9) described a novel 90-kD nucleolar protein (NOR-90) that is exclusively localized to NORs in dividing cells and to nucleoli in interphase. Interestingly, scleroderma patients also make autoantibodies to RNA polymerase I (5), DNA Topoisomerase I (10), and fibrillarin (11). In this study, our initial objective was to characterize the NOR-90 autoantigen. Our approach was to obtain cDNA clones for this nucleolar protein using the human autoantibody probe.

[1] *Abbreviation used in this paper:* NOR, nucleosus organizer region.

Materials and Methods

Antibody and Immunoblotting. Human anti-NOR sera were collected over the last 2 yr at the W.M. Keck Autoimmune Disease Center Laboratory serum bank. Anti-NOR-90 sera JO and SC were kindly provided by Professor M. J. Fritzler, (University of Calgary, Alberta, Canada). Reference anti-NOR-90 serum CAG from the original study was also obtained from Dr. C. Gelpi (Santa Cruz y San Pablo Hospital, Barcelona, Spain) (9). MOLT-4 cell extracts were prepared and separated on SDS-PAGE using a 20-cm 15% separating gel and analyzed by immunoblotting using sera diluted 1:100 and ^{125}I-protein A (ICN Biochemicals, Irvine, CA) as detecting reagent (12). Affinity-purified antibodies to recombinant phage plaques were prepared by incubating diluted serum ST with recombinant phage protein bound to nitrocellulose filters and subsequently eluted at pH 2.3 as described (12).

Immunofluorescence. HEp-2 cells were cultured in DMEM as described (13), grown on coverslips, fixed for 20 min at room temperature in 2% formaldehyde buffered with PBS, and permeabilized with 100% acetone at −20°C. Human sera were used at 1:100 dilution. Fluorescein-conjugated goat anti-human IgG was used as secondary detecting reagent. For chromosomal spreads, male Indian muntjac cells (cell line CCL 157), cultured in HAM's F-10 medium, were treated with Colcemid (0.01 μg/ml) for 6 h, and mitotic cells were processed for immunofluorescence (14). Before mounting, chromosomes were counter-stained with ethidium bromide (1 μg/ml). Silver staining for NORs was performed as described (15).

cDNA Cloning and Sequence Analysis. Human serum ST was

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used for immunoscreening of 10^6 recombinants of a MOLT-4 λgt11 cDNA library (16). Two clones, J1 and J3, were selected and cDNAs were subcloned into the EcoRI site of pBluescript SK$^-$ (Stratagene, La Jolla, CA). A HepG2 cell λZap cDNA library (gift from Dr. Frank R. Jirik, University of British Columbia) was screened for full-length NOR-90 cDNA clones by DNA hybridization using two overlapping complementary synthetic oligonucleotides (5′-TGGCCCGATTCAGGGAGGATCACCCC-GACC-3′ and 5′-TGGCATTCTGGATTAGGTCGGGGTGAT-CCT-3′) designed from the 5′ sequence of the J3 cDNA. These primers were mixed and labeled with [^{32}P]ATP using the standard fill-in reaction of Klenow polymerase (17). Clones NOR2 and NOR5 were selected and subcloned in vivo into pBluescript plasmid using R408 helper phage (Stratagene), as recommended in the manufacturer's instructions. Plasmids were purified and DNA sequences in both strands were determined (18, 19). DNA and protein sequences were analyzed by the Genetics Computer Group Sequence Analysis Software Package for VAX computers (20). Alignment of protein sequences was initially achieved with the GAP program that used the algorithm of Needleman and Wunsch (21). Multiple sequence alignments were performed with CLUSTAL programs (22, 23).

Immunoprecipitation of Recombinant and Cellular Products. Plasmid clone NOR5 and a hUBF cDNA plasmid pTβGUBF1, which was kindly provided by Drs. H.-M. Jantzen and R. Tjian (24), were used as DNA templates. RNA was transcribed in vitro from linearized plasmids using T3 or T7 RNA polymerase and was translated in vitro in a rabbit reticulocyte lysate (Promega Biotec, Madison, WI) in the presence of [^{35}S]methionine (Tran^{35}S-label; ICN Biochemicals), as described in the manufacturer's instructions. These labeled in vitro products were used as substrates in an immunoprecipitation assay (13). HeLa cells were cultured as monolayers and labeled with [^{35}S]methionine as described (13). Labeled HeLa cells were harvested by lysis in buffer A (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.5% NP-40) on ice and centrifuged at 10,000 *g* for 10 min. The nuclear pellet was sonicated in 0.1% SDS, 0.5% NP-40, 0.1% deoxycholic acid, 50 mM Tris-HCl, pH 7.5, by three 10-s bursts. The resulting nuclear supernatant was used as substrate in immunoprecipitation assay and analyzed by SDS-PAGE using a 10% separating gel (13).

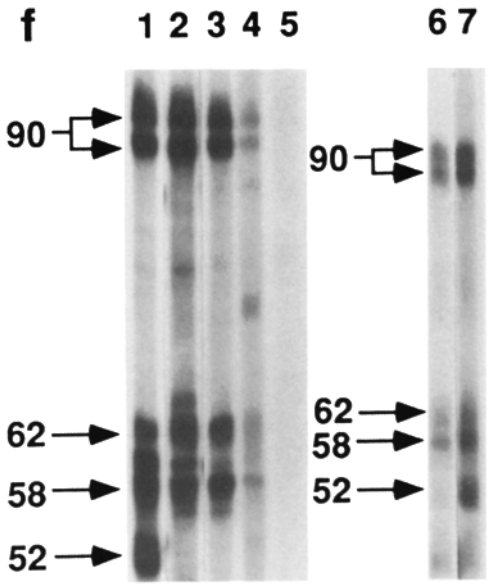

Figure 2. Immunoblotting analysis of MOLT-4 cell extracts. Lanes *1–4*, human NOR-90 autoimmune sera ST, II, JO, and SC; lane *5*, a normal human serum; lane *6*, affinity-purified antibodies from λJ3 plaques reacted with serum ST showing the reactivities with the 90-kD proteins and the 58- and 62-kD bands; lane 7, whole serum ST control. Note the affinity-purified antibody preparation no longer recognized the 52-kD SS-A/Ro protein (lane *6*).

Results and Discussion

The anti-NOR-90 sera used in this study gave strong NOR immunofluorescence in dividing HEp-2 cells, and, in interphasic nucleoli, discrete speckles were seen resembling nucleolar fibrillar centers that are active in rDNA transcription (Fig. 1 *a*). The immunofluorescence staining of NORs could be particularly well demonstrated in male Indian muntjac cells that have a diploid chromosome number of 7 (25) with two NORs located on the X and Y_1 chromosomes (Fig. 1 *b*).

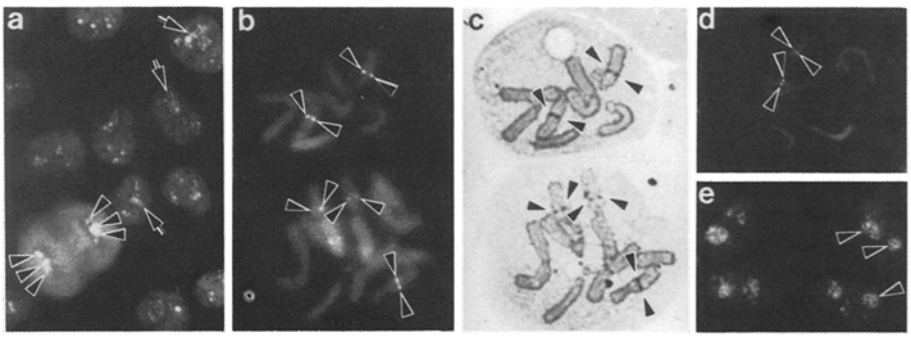

Figure 1. Immunolocalization of NOR-90 proteins. (*a*) HEp-2 cells were stained with human NOR-90 serum II showing NOR fluorescence (*arrowheads*) in dividing cells. Note the multiple discrete speckles in interphase nucleoli (*arrows*). (*b*) Chromosomal spreads of Indian muntjac cells reacted with serum II to show NOR staining (*arrowheads*). Chromosomal staining observed was produced by ethidium bromide counterstaining. (*c*) The same substrate in *b* was later processed with the NOR silver staining method to visualize NORs (*arrowheads*). (*d*) The NORs (*arrowheads*) of Indian muntjac chromosomal spreads were stained by affinity-purified antibody to the recombinant protein derived from the NOR5 clone. (*e*) HEp-2 cells showing discrete speckles in interphase nucleoli were stained with affinity-purified antibodies to the recombinant fusion protein encoded by the λJ3 clone (×160).

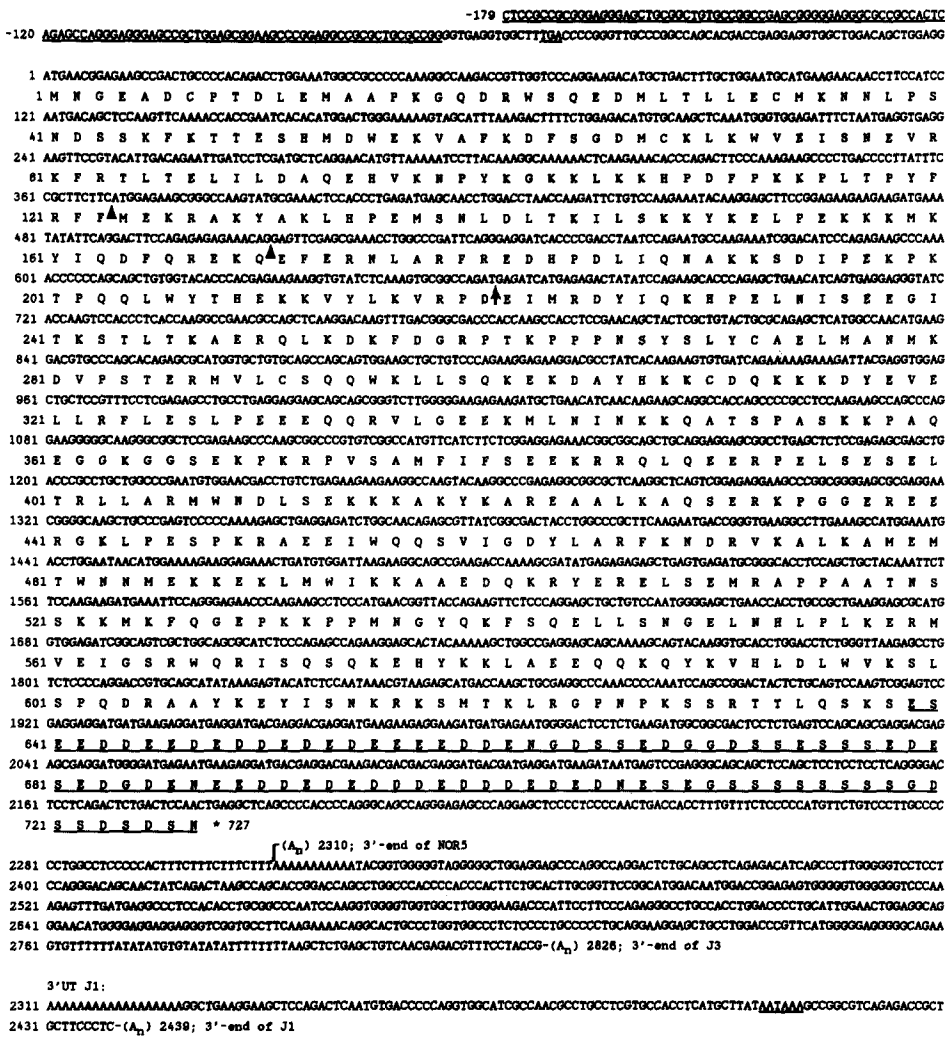

Figure 3. The complete nucleotide/protein sequence (NOR5/J3 combined) and the 3'UT sequence in J1 shown here have been submitted to EMBL/Genbank under accession numbers X56687 and X56688, respectively. The 5' UT sequence, which differed from that of hUBF, an in-frame stop codon in the 5'UT, an AATAAA polyadenylation signal sequence in the 3'UT of J1, and the COOH-terminal acidic region are underlined. Two arrowheads indicate the 5'-start of clone J1 and J3, respectively. The sequence of the four independent NOR cDNAs all lack 111 nucleotides reported in hUBF (24). The arrow indicates the site of the deletion of 111 nucleotides (37 amino acids), which is present in hUBF.

The location of NORs was confirmed by silver staining (Fig. 1 *c*). Fig. 2 shows the immunoblotting analysis of human NOR-90 sera. All NOR-90 sera showed common reactivity to two bands in the 90-kD region and to other bands at 58 and 62 kD. This separation of NOR-90 into a doublet is apparent (Fig. 2; reference 26), although this was not clearly resolved in the earlier report (9). The 58- and 62-kD species were probably degradation products of the 90-kD proteins since they were also recognized by affinity-purified antibodies (see below). Furthermore, the relative intensity of reactivity within these bands was constant over a 250-fold dilution range of serum. Autoantibody titers were generally very high; NOR-90 bands were detectable with 1:25,000 dilution of serum JO. The four human anti-NOR-90 sera did not have other detectable autoantibody specificities, except serum ST, which had autoantibodies to the 52-kD SS-A/Ro protein autoantigen (12) (Fig. 2, lanes *1* and *7*).

Serum ST was used to screen a MOLT-4 cell λgt11 library, and two cDNA clones, λJ1 (2 kb) and λJ3 (2.3 kb), were isolated. To show that these clones encoded the NOR-90 proteins, antibodies were affinity purified from serum ST using recombinant plaques bound on nitrocellulose. Affinity-purified antibodies to λJ1 or λJ3 plaques reacted with NORs (Fig. 1 *d*) and demonstrated expected patterns of speckled staining in interphasic nucleoli (Fig. 1 *e*). The same affinity-purified antibody was specific for NOR-90 proteins in immunoblotting (Fig. 2, lane *6*). The nucleotide sequences of J1 and J3 were identical, except at the 3'-untranslated (3'UT) regions (Fig. 3).

Northern blot analysis of cellular mRNA showed that J1 and J3 cDNA inserts detected a major band at ~3 kb and a minor band of higher M_r, and were therefore too small to encode the complete mRNA. Two clones, NOR5 (2.5 kb) and NOR2 (1.2 kb), were further selected from a HepG2 cell cDNA library using 5' oligonucleotide probes from J3 cDNA. Although the 3' UT region was mostly absent in clone NOR5 (Fig. 3), sequence analysis of NOR5 cDNA showed an open reading frame spanning 727 amino acids, and the predicted protein had a pI of 5.3 (20) and a molecular mass of 84,842 daltons. In vitro transcription and translation

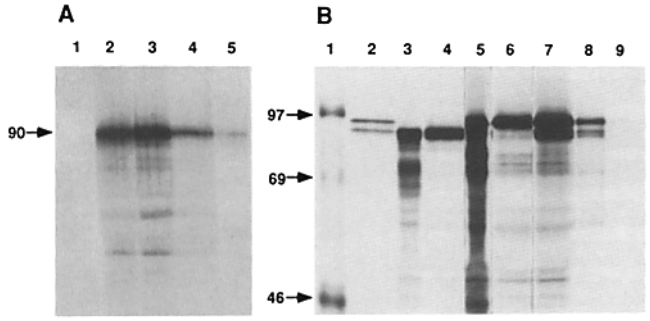

Figure 4. Immunoprecipitation analysis. (*A*) The [^{35}S]methionine-labeled in vitro translation products of NOR5 were tested for reactivity with human sera. Lane *1*, normal human serum; lanes *2–5*, human anti-NOR-90 sera ST, II, JO, and SC, respectively. (*B*) Comigration of NOR-90 proteins with NOR5 and hUBF in vitro translation products. Lane *1*, ^{14}C-labeled molecular mass markers (New England Nuclear, Boston, MA). Lane *2*, immunoprecipitation of ^{35}S-labeled HeLa cell extracts with serum JO. Lanes *3* and *4*, ^{35}S-labeled in vitro translation products derived from NOR5 cDNA before and after immunoprecipitation with serum JO, respectively. Lanes *5* and *6*, ^{35}S-labeled in vitro translation products derived from hUBF cDNA plasmid pTβGUBF1 before and after immunoprecipitation with serum JO. Lanes 7, *8*, and *9*, the immunoprecipitation of an equal mixture of the labeled in vitro translation products of hUBF and NOR5 using serum JO, affinity-purified antibody from λJ3 plaques, and a normal human serum, respectively.

of NOR5 cDNA yielded a 90-kD protein recognized by all anti-NOR-90 sera (Fig. 4 *A*).

Homology search in the GenBank/NBRF database showed that the NOR protein sequence was identical with that of rRNA transcription factor hUBF (24), with the exception that the published hUBF sequence had an additional 37-amino acid insert (Fig. 3). Since rRNA accounts for ~80% of total cellular RNA, the genes for this important RNA species have been studied extensively (for review see reference 27). Efficient rRNA transcription requires the presence of RNA polymerase I complex and at least two auxiliary factors, SL1 and UBF (28). hUBF is known to bind to an upstream control element (−200 to −107) and a core element (−45 to 20) of rRNA genes (29, 30). Footprinting data suggest that hSL1 and hUBF form a protein-protein complex that interacts with a critical region of the upstream regulatory sequence (31). The formation of the hSL1-hUBF complex is essential for the activation of rRNA transcription, and the upstream control element is known to enhance the transcriptional activity of RNA polymerase I by 10–100-fold (24).

Purified cellular hUBF was reported to consist of two polypeptides of 94 and 97 kD (31). Direct comparison of the in vitro transcription and translation products of NOR-90 and hUBF cDNAs (24) showed that the product of NOR5 comigrated with the lower band of the NOR-90 doublet while the product of hUBF had the same gel mobility as the upper band of the NOR-90 doublet (Fig. 4 *B*). The recombinant hUBF protein was also immunoprecipitated by the NOR-90 human autoantibodies. Further proof that NOR-90 and hUBF are identical was provided by affinity-purified antibodies from λJ3 plaques, which recognized both proteins (Fig. 4 *B*, lane *8*).

Southern blot analysis of human PBL genomic DNA suggested only one gene for NOR-90 cDNA (data not shown and reference 24). Therefore, we propose that the two proteins are derived from a common precursor hUBF mRNA via alternative splicing (Fig. 5 *a*). The analysis of hUBF protein sequence revealed motifs (HMG box) that were similar to the high mobility group (HMG-1) nonhistone chromosomal proteins (24). When UBF was used as the prototype HMG box protein, several interesting members of this DNA binding protein family were described (see reference 32). Our sequence alignment analysis showed that there were six HMG boxes in the high M_r form of hUBF, while in the low M_r form, the central 37 amino acid residues of the second HMG box (II) were absent (Fig. 5 *b*). Comparison of the six HMG boxes showed that HMG boxes I, II, and III had the highest degree of similarity to boxes IV, V, and VI, respectively (Fig. 5 *c*). In the earlier study (24), boxes V and VI were not described but they actually have higher percent identity values (26.6% and 27.1%) to human HMG protein than boxes II and III (21.2% and 21.7%; Fig. 5 *c*). A recent report in the Xenopus UBF sequence also suggested that there were two extra unreported HMG boxes (V and VI) in the hUBF sequence (33). Since Jantzen et al. (24) showed that the recombinant fragment containing residues 1–204, including HMG box I, was as efficient in DNA binding as the full-length hUBF molecule, the alternative form described here might be expected to recognize and bind to the UBF recognition sequence efficiently.

Jantzen et al. (34) reported a single HeLa cDNA that included HMG box II, while we obtained from two cDNA libraries four independent cDNA clones all lacking the 37-residue sequence. The two forms of UBF have been detected in several species, including rat and frog (34, 35). The NOR-90 doublet was also detected in approximately equal intensities in HeLa, MOLT-4, and rat Novikoff hepatoma whole cell extracts by immunoblotting (data not shown). Our cloning of the low Mr hUBF form was in complete agreement with a recent report by O'Mahony and Rothblum (36), who described a similar 37-amino acid in-frame deletion in the low M_r form of rat UBF.

Secondary constrictions are normally identified as achromatic regions on prophase or metaphase chromosomes that are stained with dyes such as Giemsa. However, electron microscopic examination of achromatic regions shows no real constriction of the chromosome since the measured widths are the same at the secondary constrictions and other regions of chromosome arms (37, 38). Therefore, the metaphase NOR, although described as a secondary constriction, may actually not be a tightly condensed region of chromosomes (37, 38). The immunolocalization of RNA polymerase I (5) and hUBF to NORs during mitosis suggests that these crucial proteins are probably never completely disengaged from rDNA even when rRNA synthesis is minimal.

Human serum autoantibodies from patients with systemic autoimmune diseases have been invaluable research tools for many studies in cellular and molecular biology (39). Although the mechanism for the production of these autoantibodies is still unclear, the striking feature is that these antibodies inhibit the functions of their respective target antigens in most

Box	I	II	III	IV	V	VI	hHMG
I	100	25.3	26.8	32.9	26.0	18.8	30.7
II		100	23.5	22.8	27.2	20.0	21.2
III			100	22.1	18.5	35.3	21.7
IV				100	18.9	20.0	30.5
V					100	17.9	26.6
VI						100	27.1

Figure 5. Features of NOR-90/hUBF. (*a*) Diagrammatic summary of NOR-90/hUBF proteins. The protein sequence of NOR5 is identical with that of hUBF except for a deletion of 37 amino acids starting at residue 221. The proposed scheme of alternative splicing may explain the relationship of the two forms of NOR-90/hUBF proteins. The HMG boxes marked with an asterisk were previously defined in hUBF (24). Boxes V and VI are defined based on the amino acid sequence alignment shown in *b* comparing hUBF and human HMG-1 sequences (hHMG). HMG boxes I, II, and III are aligned adjacent to boxes IV, V, and VI, respectively, in order to show their respectively closer relationships. Filled and open circles highlight the identical amino acid residues and conserved substitutions between the subgroups: boxes I, IV; II, V; III, VI. Conserved or identical amino acids in four or more of the seven listed sequences are boxed. Conserved amino acids are grouped as follows: (K,R,H), (E,D,Q,N), (S,T), (F,Y,W), (G,A,P), and (M,L,V,I). Sequence in hUBF HMG box II that is absent in the NOR5 protein sequence is double underlined. (*c*) The percent identity derived from the GAP program is listed for comparison among the HMG boxes of hUBF. HMG boxes I, II, and III show the highest degree of identity to boxes IV, V, and VI, respectively.

functional assays (39). Therefore, our description of antibody to a RNA polymerase I transcription factor may provide new tools for the functional study of rDNA gene regulation. The definition of NOR-90 antigen as an accessory factor in RNA polymerase I transcription also adds to our understanding of the molecular structure and function of autoantigens.

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