Nucleic Acids Research, 1993, Vol. 21, No. 7 1581–1585

Different TRE-related elements are distinguished by sets of DNA-binding proteins with overlapping sequence specificity

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Received January 18, 1993; Revised and Accepted February 26, 1993

ABSTRACT

Several promoter elements with sequence similarity to the prototype TPA-responsive element (TRE) were compared by mobility-shift analyses. Activities within whole cell extracts were identified that bind to the TRE-like elements in the collagenase, the somatostatin, and the c-*jun* promoters. The corresponding factors appeared to differ in their degree of selectivity for these TRE-like sequences. One protein species bound equally well to all TREs. In addition, a subset of specific activities recognised only the somatostatin and the c-*jun*-derived element and one DNA – protein complex had exclusive specificity for the TRE present in the c-*jun* promoter. By antibody 'supershift' assays some of the protein components of the specific complexes were identified as CREB- and ATF-related products. Based on these data we postulate that bZip protein dimers differ in their ability to tolerate variations from the canonical TRE sequence. We propose that TRE-like promoter elements are distinguished by this ability to bind to different subsets of a family of related transcription factors.

INTRODUCTION

Promoters are composed of discrete *cis*-acting sequence motifs which represent the binding sites for specific transcription factors and determine the basal and inducible transcription rates of a linked gene (1, 2). These DNA sequence elements can, for example, confer transcriptional inducibility of genes by signals such as exposure to heat shock or heavy metals, viral *trans*-activators, elevated levels of cAMP, and a variety of natural and synthetic mitogens. Gene activation in response to cAMP and the tumour-promoting agent and protein kinase C agonist 12-O-tetradecanoyl phorbol 13-acetate (TPA) can be mediated by two closely related DNA sequences, the cAMP- and TPA-response elements (CRE and TRE), respectively. The TRE consensus sequence has been defined as a short palindrome {TGA($^{C}/_{G}$)TCA} which differs from the CRE consensus (TGACGTCA) by only one base pair. The homology between these sequence elements is mirrored by the similarity of the cognate sequence-specific DNA binding proteins. These factors are members of the bZip family. Proteins belonging to this class possess a common domain consisting of a basic region that contacts the DNA, and an adjacent leucine zipper domain which mediates the dimerisation obligatory for DNA binding. (3, 4). Heterodimers between Fos- and Jun-related bZip proteins or Jun homodimers are known to bind with high affinity to TRE sequences. It is these heterodimeric complexes which appear to elicit the induction of many TPA responsive genes (5). The cAMP-responsive character of CREs is effected by another class of bZip proteins composed of the CREB-related factors, although the CRE also represents the target binding site for the ATF sub-family. The distinction between CRE and TRE sequences on the one hand, and of Jun/Fos and CREB/ATF factors on the other is, however, not absolute. It is apparent that TRE- and CRE-related sequences exist that are recognised by both groups of bZip proteins (6, 7). Cross-talk between these categories of bZip proteins also occurs at the protein level; heterodimers composed of c-Jun and some members of the ATF/CREB families e.g. ATF-2, -3, -4, and CREB have been reported, and appear to preferentially bind to CRE sequences (8, 9).

In this paper we have sought to focus on the following idea: Can such variability be better accommodated not by defining independent *cis*-acting transcription control elements (TREs and CREs) with separate cognate binding factors, but instead by considering a spectrum of related binding motifs which exhibit subtly different (and sometimes overlapping) activities? This question has been addressed with a study of the binding activities by mobility-shift assay present within whole cell extracts derived from CCL64 (mink lung) cells, to several naturally occurring promoter elements. These motifs contain consensus and variant TRE and CRE sequences which are reported to have different regulatory functions. These elements (shown in Table 1) are: (i) The 'classical' TRE derived from the collagenase gene promoter (coll TRE) (5, 10), (ii) The TRE in the promoter of the *c-jun* gene, which carries an additional base pair compared to the previous sequence (jun TRE) (11). The jun TRE has been shown to be responsive to TPA but also to the adenoviral protein E1A (11, 12, 13). This site is thought to mediate autoregulation of the *c-jun* promoter; (iii) The 'classical' CRE derived from the

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somatostatin gene promoter (CRE) (14). Our results indicate that there are factors within the extracts which bind each of the sequence motifs studied. It appears, however, that there are proteins more restricted in their ability to recognize certain elements. We suggest that TRE-like sequences in general will be recognised by common factors. However, only some proteins will be able to tolerate subtle nucleotide variations. In this way an element may retain responsiveness to a particular signal, but can possess additional regularity characteristics.

MATERIALS AND METHODS

Cell culture and preparation of whole cell extracts

CCL64 cells were grown in DMEM supplemented with glucose (4.5g/l), 10% fetal calf serum, 2 mM glutamine, 10 μg/ml penicillin, and 10μg/ml streptomycin. Approximately 1×10^6 cells were seeded into 10 ml of media in 10 cm culture dishes 12−16 h prior to harvesting. Whole cell extracts were prepared based on a method developed by Zimarino and Wu (15). Briefly, cells growing in 10 cm culture dishes were washed twice with ice-cold PBS. The cells were scraped off the plate with a rubber policeman in 1 ml of PBS into an Eppendorf tube and sedimented by a 5″ spin at 4°C. The supernatant was removed and the cell pellet was frozen in liquid nitrogen. The cell pellets were then either stored at −80°C or processed as follows: The frozen cells were placed on ice, 5 packed cell volumes (approximately 10 μl) of ice-cold extraction buffer was added (10 mM HEPES, pH 7.9, 0.4 M NaCl, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol, 0.5 mM PMSF). The cells were resuspended and lysed by pipetting up and down in the tip of a pipetman until the mixture appeared homogeneous. Cell debris was removed by centrifugation at 12,000 rpm in a microcentrifuge for 15′ at 4°C. The lysate was carefully transferred into a pre-chilled Eppendorf tube before being aliquoted, frozen in liquid nitrogen, and stored at −80°C. Protein concentration was determined by the Bradford assay.

Electrophoretic DNA-binding assays

The sequences of the oligonucleotides utilized in the mobility-shift assays and the copper-phenanthroline (Cu-OP) foot-printing technique are shown in Table 1. The probes for the mobility-shift assays were produced by annealling each of the oligonucleotides shown in Table 1 to a primer with the sequence 5′-CGTCCAGTCGAGC-3′, and labelled by filling-in with deoxyribonucleotide triphosphates including α^{32}P-dCTP and DNA polymerase I (Klenow fragment) (16). For the Cu-OP procedure, the indicated oligonucleotides were cloned into Bluescript KS+ vector (Stratagene) in order to generate probes of the required length (17). The resultant plasmid was linearized, ~2 pmoles of 5′ ends were end-labelled with γ^{32}P-ATP and T4 polynucleotide kinase, the DNA recut and the labelled fragment gel purified. The DNA-binding reaction volumes were 20 μl and the following reagents were mixed in this order: 2 μl 10×binding buffer (200 mM Tris.Cl, pH 7.5, 750 mM KCl), DTT to a final concentration of 1 mM, 5 μg BSA, 1 μg poly dI-dC, water, 5 μg whole cell extract protein, and 7−10,000 cpm (Cerenkov) of radioactively labelled probe (approximately 2 fmoles of DNA).

Table 1. A comparison of various TRE-like sequences

Probes	TRE-like elements
coll TRE: 5′	tacaggtacgA TGA_GTCA Ggctcgactggacg 3′
jun TRE:	tacaggtacgG TGACATCA Tgctcgactggacg
CRE:	tacaggtacgC TGACGTCA Ggctcgactggacg
Primer:	3′ cgagctgacctgc 5′

The oligonucleotide sequences utilized in the gel retardation assay are shown. The sequences of interest derived from the indicated promoters are represented in capitals and the TRE-like sequences themselves are boxed. The orientation of each of the binding sites within the respective promoters corresponds to that directed towards the transcription initiation site. The remainder of the oligonucleotide (lower case letters) is randomly generated and contains no additional binding sites characterized to date. 'Primer' refers to the sequence which is annealled to each of the oligonucleotides allowing the synthesis of the labelled mobility-shift probes using the 'fill-in' procedure (described in Materials and Methods).

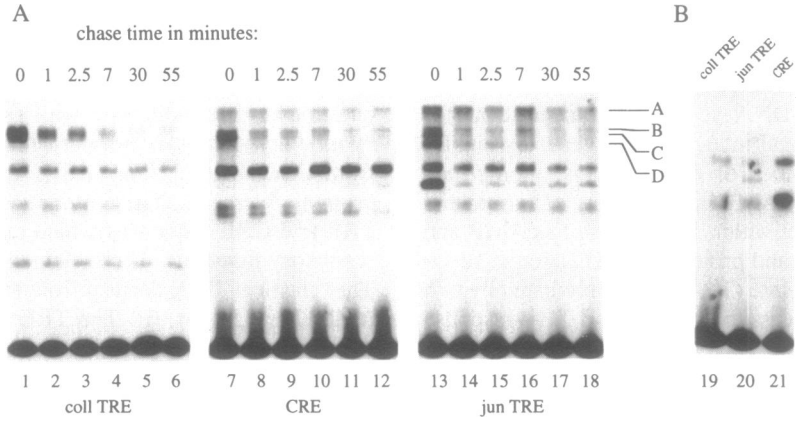

Figure 1. (A) Dissociation rate analysis of the complexes formed by the various TRE-like elements [lanes 1−6, the collagenase TRE (coll TRE); lanes 7−12, the somatostatin CRE (CRE); lanes 13−18, the TRE-like site within the *c-jun* promoter (jun TRE)]. The binding reactions were allowed to reach equilibrium and were then chased with an excess of the corresponding unlabelled oligonucleotide for the incubation times indicated such that all samples were loaded concomitantly. A, B, C and D on the right of the panel point to the different complexes discussed in the text. (B) Binding competition analyses employing the indicated oligonucleotides as radioactive probes and as homologous competitors.

The reaction was incubated at room temperature for 25'. DNA competitor was added where indicated 5' prior to the radioactive probe. For dissociation rate analysis, 2 μl of each of the indicated competitors (300 fmoles/μl in 1×binding buffer) was added at the end of the binding reaction and incubation was continued for the specified periods. Antisera for 'supershift' analyses were added to the binding reaction and incubated for 30' at room temperature prior to the addition of the probe. A 5% non-denaturing polyacrylamide gel was pre-run at 20 mA for 25' in 0.25×TBE buffer (22.5 mM Tris-borate, 0.25 mM EDTA, pH 8.0). Samples were loaded and the gel was run for 3 h at constant current (20 mA). Cu-OP footprinting was carried out according to the procedure of Kuwabara and Sigman (18).

Antibodies

α-CREB peptide antibody raised against the carboxy-terminal ten amino-acids of CREB was a generous gift from Dr K.Lee, ICRF, London(19). α-ATF peptide antibodies raised against amino acids Asp328−Arg337 of ATFa were kindly provided by Dr B.Chatton (CNRS-LGME, Strasbourg)(20).

RESULTS

Binding activities specific for different TRE-like sequences

Radiolabelled double-stranded oligonucleotides were prepared bearing the following sequences (see Table 1): the TRE derived from the collagenase gene promoter (coll TRE), the TRE-like sequence located within the c-*jun* promoter (jun TRE), and the somatostatin promoter cAMP-response element (CRE). In all cases the TRE-like sequences are flanked by an extra nucleotide which occurs in the corresponding promoter context (uppercase letters flanking the boxed TRE-like sequences). The inclusion of these extra bases can influence the stability of some complexes (21). The remaining oligonucleotide sequences (indicated by lower case letters) are identical. These labelled oligonucleotides were incubated with whole cell extracts and the resulting protein−DNA complexes were separated on non-denaturing polyacrylamide gels. These mobility-shift experiments yielded several bands, some of which were considered unspecific because they were insensitive to competition with excess amounts of the unlabelled probe (Fig. 1 and data not shown), and the faster-migrating species appeared to be degradation products. Four complexes fulfilled our criteria for specificity, namely competibility and reproducibility. These complexes, termed A, B, C and D, displayed differential specificity for the various probes. With the coll TRE probe only complex C could be detected, the CRE probe was shifted into bands A, B and C, and the jun TRE generated all four bands (Fig. 1, lanes 1, 7, and 13).The formation of complexes A, B, C, and D could be inhibited by competition with a 300 fold excess of unlabelled probe (Fig. 1, panel B).

To begin characterising the protein−DNA complexes that gave rise to bands A, B, C and D a dissociation rate analysis with the coll TRE, jun TRE, and CRE probes was carried out. The binding reactions were allowed to equilibrate under standard reaction conditions and followed by a chase with a large excess (300 fold) of the same unlabelled oligonucleotide as a specific competitor for various time intervals prior to analysis by gel electrophoresis. Proteins which dissociate from the labelled probe during the incubation time will be sequestered by the excess of the unlabelled oligonucleotide and consequently, the decrease in band intensity with the chase time reflects the relative dissociation rate of the corresponding protein−DNA complex. Fig 1 shows the result of this analysis. The rate of decay of the complexes corresponding to bands A, B, C, and D generated by each of the probes indicated did not vary significantly with the sequence of the oligonucleotide. However, a clear difference could be observed when comparing the relative dissociation rates between complexes A, B and C generated by the CRE or complexes A, B, C and D formed on the jun TRE (compare for example lanes 7−12 and 13−18 in Fig. 1). Complex A was most stable followed by complex B, whereas complex C dissociated rapidly. The stability of complex D, formed only by the jun TRE, was approximately equivalent to that of complex B. Therefore the A-, B-, C- and D-specific proteins can be distinguished on the basis of their sequence preference, the electrophoretic mobility of the corresponding complexes, and their respective relative dissociation rates. Each of these properties suggest that distinct polypeptides are involved.

To establish that all three specific complexes were formed on the TRE elements and not on flanking, non-related nucleotides, a footprinting experiment was performed on the individual complexes (Fig. 2). Preparative mobility-shift assays were carried out with asymmetrically end-labelled jun TRE and coll TRE probes carrying approximately 110 additional basepairs of plasmid DNA to generate a fragment size that is suitable for footprinting experiments (see materials and methods). The free and the protein-bound fragments were exposed *in situ* to the nucleolytic activity of copper-phenanthroline, a chemical reagent

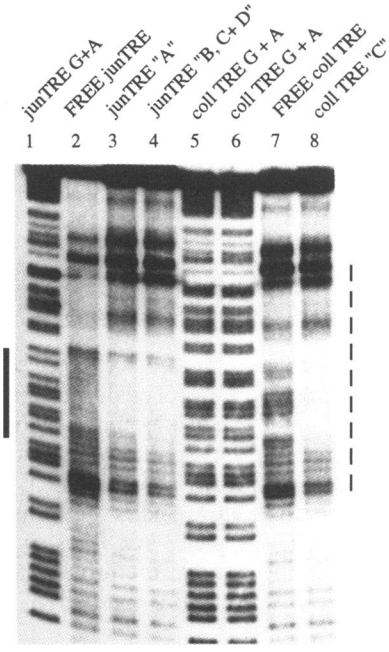

Figure 2. Copper-phenanthroline (Cu-OP) footprinting of the complexes formed by the TRE-like element within the *c-jun* promoter (jun TRE, lanes 1−4) and the collagenase TRE (TRE, lanes 5−8) following mobility-shift assays. Lane 2: *in situ* Cu-OP treatment of the free jun TRE probe; lane 3, *in situ* Cu-OP treatment of complex 'A'; lane 4, *in situ* Cu-OP treatment of complexes 'B, C + D'. Lane 7, *in situ* Cu-OP treatment of the free coll TRE probe; lane 8, *in situ* Cu-OP treatment of complex 'C'. Lanes 1, 5, and 6 are Maxam and Gilbert G+A sequencing ladders of the indicated probes. The black-filled bar on the left demarcates the protected region, and the black-dashed bar (right) corresponds to the oligonucleotide utilized in the mobility-shift analyses which was cloned in pBluescript vector for this experiment (see Materials and Methods).

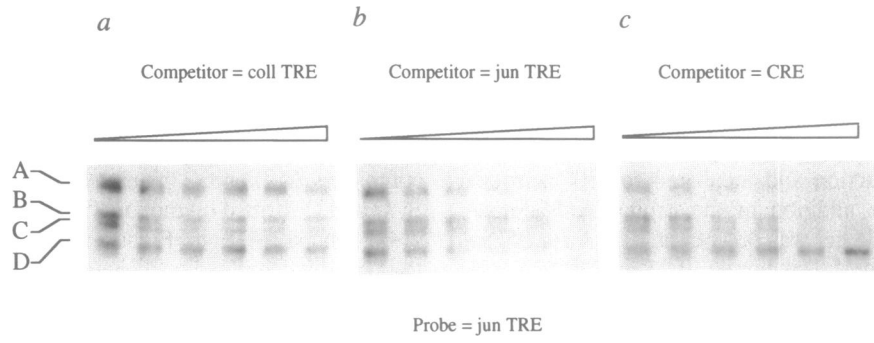

Figure 3. DNA-binding heterocompetition analysis of the complexes generated by the *c-jun* promoter TRE-like element (jun TRE). All binding reactions were performed with the jun TRE as probe in the presence of increasing amounts of the indicated unlabelled oligonucleotides as competitors (**a**, coll TRE; **b**, jun TRE; **c**, CRE). A, B, C, and D on the left mark the various complexes discussed in the text. Only the region of the gel containing complexes is shown.

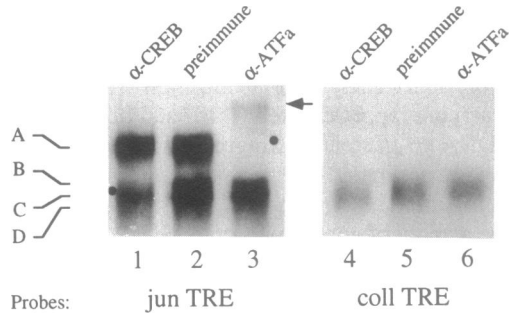

Figure 4. Immunological characterisation of some of the factors involved in complexes generated by both the *c-jun* promoter TRE-like element (jun TRE, lanes 1–3) and the collagenase TRE (coll TRE, lanes 4–6). In each case the indicated antiserum was preincubated with extract for 30′ prior to assembling the DNA-binding reaction. A, B, C and D on the left point to the different complexes discussed in the text. Black dots indicate the position of complexes that were either eliminated (lane 1, complex B) or further retarded (lane 3, complex A) as a result of antibody addition. The arrow on the right points to the 'supershifted' species.

which cleaves DNA at bases that are not protected by interacting protein (18). The treated free probes do not reveal a footprint as shown in lanes 2 and 7. Such a footprint is however obtained on both probes with the specific protein–DNA complexes. The region footprinted encompassed the entire TRE sequence in all cases as indicated in the figure, and is apparent for complexes A and B+C+D (jun TRE, lanes 3 and 4) and B (coll TRE, lane 8). Consequently, it appears that the proteins which form the different complexes specifically recognise the TRE-like sequences.

Specificity of the complexes which interact with the Jun promoter TRE

To further investigate the overlapping DNA-binding specificities analysed so far, heterologous competition experiments were carried out. The probe for the experiment shown in Fig. 3 was the jun TRE which is the only one that generates all four complexes. In the binding reactions increasing amounts of unlabelled jun TRE, coll TRE, or CRE were included. The complexes exhibited different competition profiles with the cold competitors indicated. Specifically, it appears that the collagenase TRE marginally competes for the formation of complexes B and C (panel a) while the somatostatin CRE competes for complexes A, B, and C (panel c). Only excess jun TRE, in the homologous competition reaction (panel b), interferes with the formation of all complexes. Consequently, the factor(s) participating in complex D appear to possess a specificity unique for the *jun* promoter site, whereas complexes A, B and C are formed equally well on the jun TRE and the CRE. Furthermore, it seems that one complex (designated C) is generated by each of the TRE-like sequences analysed here.

Immunological characterization proteins binding to the Jun promoter TRE

Many of the genes encoding proteins of the bZip class have been cloned and antibodies have been raised against the gene products (19, 20, 22, 23, 24). To obtain information about the possible constituents of complexes A, B, C and D we included antisera directed against a number of bZip proteins in the DNA-binding reactions. Antibody addition experiments carried out with anti-cJun, anti-Jun D and anti-cFos sera generated negative or inconclusive results. It seems that all probes can generate one or more complexes that contain proteins which crossreact with polyclonal anti-cJun serum. However, identification of the specific complexes affected was not possible (data not shown). Clear results were obtained with antisera directed against CREB and ATFa (Fig. 4). The anti-CREB serum specifically abrogates formation of complex B, implying that CREB is an essential protein component of this jun TRE- and CRE-specific complex. Complex A is the target for inhibition by the anti-ATFa serum. This is evident by the disappearance of band A and the generation of a new, slower-migrating 'supershifted' band which most likely represents a complex between DNA-bound ATFa and the specific antibody. Significantly, neither of these antisera affected the integrity of complex C formed on the coll TRE probe (Fig. 4, lanes 4–6). A significant difference between the coll TRE and the jun TRE therefore is the ability of the latter and not the former, to bind to ATFa and CREB. Unfortunately, none of the tested anti-sera gave an indication of the identity of the jun TRE-specific complex D (data not shown).

DISCUSSION

The TRE- and CRE-like sequences studied here differ only by single base-pair changes or insertions from each other, yet they

confer different transcriptional characteristics to linked genes. The coll TRE is inducible via the protein kinase C pathway, i.e. by TPA or certain activated oncogenes, but is generally not responsive to elevated intracellular levels of cAMP (6). The somatostatin CRE on the other hand can stimulate transcription of a *cis*-linked gene if increased cAMP concentrations activate protein kinase A in the target cell. The jun TRE responds to TPA but also to E1a and retinoic acid in transient transfection studies (25). The data presented here suggest the following hypothesis: the unique regulatory characteristics of the related *cis*-acting transcription control elements analysed in this work might not be mediated by separate binding activities unique for any one of the sequence motifs. Rather, it is the particular set of factors capable of binding to a certain genetic element that determines its regulatory identity. Hence the factors which are involved in this process differ not in absolute sequence specificity, i.e. they have common preferences for 'strong binding sites' (like the jun TRE), but they vary in the extent to which this specificity is restricted, i.e the spectrum of sequences they recognise. Such a system may arise if a number of related transcription factors evolve from an ancestral bZip protein that differ only in their ability to recognise variants of a given prototype sequence. Consequently, highly related sequences may be bound by a common set of factors but additional sub-populations of distinct complexes may also be generated which would be dependent upon the subtle sequence deviations. Interestingly, this appears to be the situation for the jun TRE relative to the CRE and the coll TRE. In this case, in addition to the common complexes formed by the coll TRE and CRE, a unique complex has been generated involving a sub-population of factors. Therefore there is a distinct profile of factors which bind to any one sequence motif. This analogy may also apply to other bZip groups such as the C/EBP family. C/EBP itself binds all the sites that are recognised by DBP plus several additional ones not recognised by DBP (26).

Due to the close similarity of the coll TRE, the jun TRE and the CRE we suspect that the activities that give rise to the four complexes described above belong to the bZip category. At our level of analysis it is not clear what the molecular kinship of the factors involved is. They could consist of different gene products. It is however also quite possible, given the dimeric nature of bZip transcription factors, that some of these four activities have protomers, for example c-Jun, in common. Further molecular and/or immunological characterisation is necessary to elucidate this point. In the studies presented here it is shown that the factors involved in the formation of complex B contain CREB or a related cross-reacting species. This interpretation is supported by the fact that complex B is not formed on the non cAMP-responsive coll TRE. Interestingly, we could ascribe the formation of complex A to ATFa or a closely related protein. It is noteworthy that ATFa and the highly related ATF-2 protein have been shown to interact with E1a and to synergize with the viral protein in transcriptional activation (27, 28). Complex A might therefore represent a protein–DNA complex within the cell necessary to mediate the described E1a regulation of c-*jun* transcription.

The experiments shown in this paper were all performed with CCL64 extracts. Qualitatively similar results were obtained when HeLa or 3T3 cell extracts were used. It seems however, that the ratio between different complexes varies with cell-type and growth state. It is intriguing to speculate that such protein-binding DNA sequences, especially those exhibiting complex binding activities and diverse patterns of control, might confer discrete regulatory characteristics to the linked gene which are influenced by the particular cellular context and the ratio of the protein factors present.

ACKNOWLEDGEMENTS

We thank B.Chatton, K.Lee and R.Bravo for generously providing antibodies and R.de Martin for advice in the cell culture of CCL64 cells. Filip Lim and Iain Mattaj helped us to improve the quality of the manuscript.

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