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Short Contribution

Cortical rotation is required for the correct spatial expression of *nr3, sia* and *gsc* in *Xenopus* embryos

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ABSTRACT **β-catenin, a component of the wnt-signal-transduction pathway, is essential for the formation of the dorsal axis in *Xenopus laevis* embryos. On the dorsal side of the embryo, β-catenin is translocated into the nuclei via a process linked to cortical rotation. When cortical rotation is blocked by UV-irradiation, nuclear β-catenin is found in the vegetal pole of the embryo. Here we show that overexpression of β-catenin in animal cap explants, in the absence of mesoderm induction, is sufficient to activate the expression of genes with dorsalizing activity such as *siamois* (*sia*) and *nodal-related 3* (*nr3*) but not *goosecoid* (*gsc*). In embryos ventralized by UV-treatment, the expression of the dorsal-specific genes *sia*, *nr3* and *gsc* is induced at the vegetal pole after the Mid-Blastula-Transition (MBT). While *nr3* and *sia* expression continues in these embryos until gastrula stages, *gsc* transcription cannot be maintained. We propose that the spatial separation of the expression domains of genes with dorsalizing activities and the prospective mesodermal region results in the loss of dorsal structures in the embryo. The role of cortical rotation is to generate an overlap of the region with dorsal axis-forming activity, indicated by nuclear translocation of b-catenin, and the prospective mesoderm in the marginal zone to assure the correct positioning of the Spemann organizer.**

KEY WORDS: *Xenopus laevis, axis formation, cortical rotation, nr3, sia, gsc, β-catenin*

Within the first hour after fertilization, the cortex of the *Xenopus* egg rotates by 30° and this process establishes the dorsoventral polarity in the egg. Cortical rotation is microtubule-dependent and can be effectively inhibited by UV-irradiation of the egg's vegetal pole shortly after fertilization. The UV-treated embryos fail to develop dorsal axis structures and differentiate into ventral mesoderm (Scharf and Gerhart, 1983). Recently, it has been demonstrated that components of the wnt-signaling pathway are essential for initiation of the dorsal program. One component of the wnt-signaling cascade that has been studied extensively is β-catenin, the vertebrate homolog of the *Drosophila* armadillo protein (Miller and Moon, 1996). Gain and loss of function experiments demonstrated that β-catenin is required for axis formation in *Xenopus* (Heasman *et al.*, 1994; Funayama *et al.*, 1995). Cytoplasmic β-catenin accumulates on the dorsal side of the early embryo and enters the dorsal nuclei together with the transcription factor XTcf-3 at the blastula stage (Molenaar *et al.*, 1996; Rowning *et al.*, 1997). Little is known about the target(s) of the nuclear β-catenin-XTcf-3 complex, however it was recently reported that *siamois (sia)*, a homeobox gene with dorsalizing activity, is activated by components of the wnt-pathway including β-catenin (Brannon and Kimelman, 1996).

Overexpression of ß-catenin induces the expression of nr3 and sia in the absence of mesoderm induction

In this study we analyzed the temporal and spatial expression profiles of dorsal-specific genes in untreated and UV-irradiated *Xenopus* embryos and asked whether β-catenin is directly involved in their activation. The genes selected for these experiments were the homeobox gene *goosecoid* (*gsc*), which is a direct target of activin and is expressed in the deep layer of the organizer (Cho *et al.*, 1991), *siamois* (*sia*), a paired-type homeobox gene that is expressed in the dorso-vegetal region but cannot be induced by growth factors (Lemaire et al., 1995) and *nodal-related 3* (*nr3*), a member of the TGFβ family of growth factors that is expressed in the epithelial layer of the organizer (Smith *et al.*, 1995). The products of these genes have axis-inducing activity and are able to dorsalize mesodermal tissue. An RT-PCR analysis demonstrated that in UV-treated embryos at stage 10.5, *gsc* expression is eliminated and the levels of *nr3* and *sia* are reduced compared to untreated control embryos (Fig. 1A). In contrast to this, the expression of *brachyury* (*bra*), a gene expressed in the dorsal and ventral marginal zone, remains unaffected by UV-treatment (Smith *et al.*,

Abbreviations used in this paper: RD-PCR, reverse transcription- polymerase chain reaction; bFGF, basic fibroblast growth factor; H4, histone H4.

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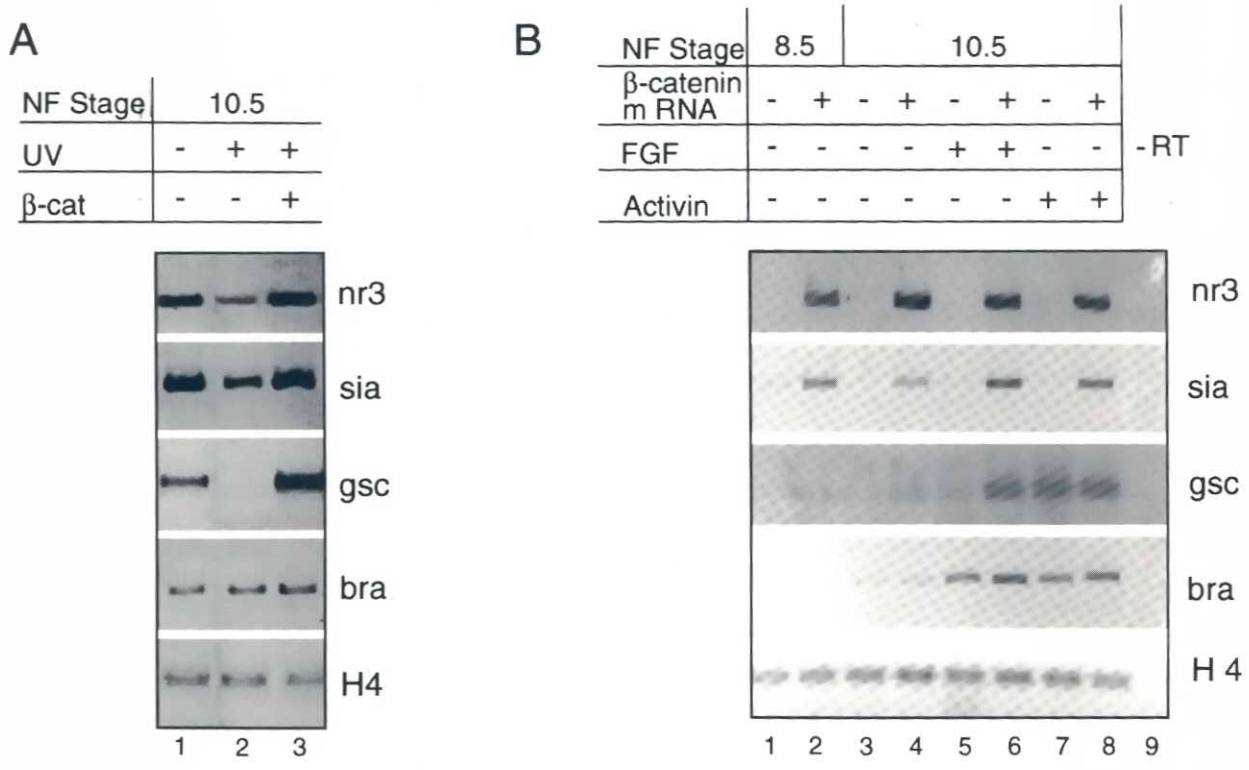

Fig.1. Activation of *sia, nr3* and *gsc* after overexpression of ß-catenin. (**A**) *Xenopus embryos were UV-irradiated (lane 2, 3), injected with 500 pg synthetic β-catenin mRNA (lane 3) and grown until NF stage 10.5. The RNA was extracted and the expression of nr3, sia, gsc, bra and H4 was analyzed by RT-PCR. (lane 1) displays RNA from untreated, uninjected stage 10.5 embryos.* (**B**) *Embryos at the 2-cell stage were injected in the animal region with 300 pg synthetic β-catenin mRNA. At stage 8 the animal caps from uninjected embryos (lanes 1, 3, 5, 7) or from those injected with β-catenin mRNA (lanes 2, 4, 6, 8) were explanted and cultured until stage 8.5 (lanes 1, 2) or 10.5 (lanes 3-8). To induce mesoderm in the explants bFGF (lanes 5, 6) or activin (lanes 7, 8) was added to the culture medium. The expression of nr3, sia, gsc, bra and H4 was analyzed by RT-PCR. -RT represents a control sample in which the reverse transcription reaction was omitted (lane 9).*

1991). Overexpression of β-catenin in UV-treated embryos elevates the transcript levels of *nr3, sia* and *gsc* but not of *bra*, indicating that the axis rescue in the injected embryos (data not shown) is due to the up regulation of genes with dorsalizing activity (Fig. 1A). The finding that the expression of *nr3* and *sia* is not completely inhibited by UV-irradiation suggests that the requirements for their stable activation differ from those of *gsc*. *gsc* is expressed in the dorsal mesoderm at stage 10.5 and we asked whether the activation of this gene by β-catenin is dependent on mesoderm formation. In contrast to *gsc*, *nr3* is expressed in the epithelial layer and *sia* preferentially in the dorso-vegetal cells that do not become part of the axis, implying that their activation is independent of mesoderm induction (Lemaire *et al.*, 1995; Smith *et al.*, 1995). To test this hypothesis, β-catenin mRNA was injected into the animal region of 2-cell embryos, the animal caps were explanted at stage 8.5 and the expression of *nr3, sia* and *gsc* was analyzed by RT-PCR at stage 8.5 and 10.5 (Fig. 1B). To determine the effect of mesoderm induction, the animal cap explants were treated with bFGF or activin protein. Uninjected, uninduced animal caps did not express *nr3, sia, gsc* and *bra* but when β-catenin mRNA was injected, *nr3* and *sia* were strongly activated after MBT. In agreement with published data, bFGF and activin induced *bra* expression and *gsc* was only induced by activin (Smith *et al.*, 1992). The overexpression of β-catenin did not change the expression pattern of *bra* in FGF- and activin-induced caps. When bFGF was applied to animal caps injected with β-catenin mRNA, however, *gsc* was activated, demonstrating that mesoderm induction is required for the expression of *gsc*. These data are in agreement with previous findings that injection of *Xwnt-8* mRNA into the animal pole region resulted in *gsc* induction restricted to the prospective mesoderm in the marginal zone (Steinbeisser *et al.*, 1993). In contrast to genes like *gsc* and *bra*, which require mesoderm to be stably expressed, *sia* and *nr3* do not need the mesodermal context in order to become transcriptionally activated and overexpression of β-catenin is sufficient to trigger their expression. Both the movement of β-catenin into nuclei in response to wnt-signaling and the resulting dorsalization process are dependent on cortical rotation.

Dorsal-specific genes are activated in the vegetal hemisphere of UV-treated embryos

It was reported that nuclear β-catenin was found in cells at the vegetal pole of UV-treated embryos (Schneider *et al.*, 1996; Larabell *et al.*, 1997). The question we therefore asked was

whether the dorsal genes *gsc*, *sia* and *nr3* would be activated at the vegetal pole of UV-treated embryos. For these experiments, we used embryos at stage 8.5-9 because we found that *gsc* was activated shortly after MBT even in UV-treated embryos but that at stage 10.5 only a very small amount of gsc message was detectable (Fig. 2A). This indicates that the transcriptional activation and maintenance of the *gsc* gene are differentially regulated. In order to determine the spatial distribution of the *nr3*, *sia* and *gsc* transcripts, the embryos were manually dissected into animal cap (AC), marginal zone (MZ) and vegetal cap (VC). The total RNA was then analyzed by RT-PCR and the level of each transcript was quantified using a Phosphorimager and ornithine decarboxylase mRNA (ODC) as a standard (Fig. 2B). No *gsc* transcripts were detected in the marginal zone of UV-treated embryos whereas *gsc* was expressed strongly in untreated dorsal marginal zone samples. This finding is in agreement with *in situ* hybridization data showing the absence of *gsc* transcripts in the marginal zone of UV-treated embryos (Cho *et al.*, 1991). Transcripts of *nr3* and *sia* were found preferentially in the vegetal pole of UV-irradiated embryos compared to untreated control samples. In control experiments it was confirmed that the dorsal specific genes *nr3*, *sia* and *gsc* were not expressed in ventral marginal zones of untreated embryos and that randomly dissected marginal zone fragments from UV-treated embryos showed strongly reduced or no expression of these genes (data not shown). When the spatial distribution of *gsc*, *nr3* and *sia* transcripts was plotted as seen in Fig. 2C, it became apparent that the expression domains of these three genes shifted towards the vegetal pole as a result of UV-treatment. This result demonstrates that the dorsal specific genes *gsc*, *nr3* and *sia* can be activated at the vegetal region after MBT when cortical rotation is blocked. It also confirms the finding of Brannon and Kimelman (1996), who demonstrated that *sia* is expressed in cells at the vegetal pole of UV-treated embryos. In cells at the vegetal pole, outside the region of the prospective mesoderm, these gene products cannot execute their dorsalizing activity on the mesodermal tissue and no dorsal axis structures develop (Fig. 3). Expression of *nr3* and *sia* is maintained in UV-treated embryos until gastrula stages but the level of *gsc* mRNA decreases dramatically between stage 8.5 and 10.5 (Fig. 1A, 2A). It seems plausible that the *gsc* gene can be activated outside the mesodermal region on the vegetal pole but that the expression can not be maintained. Experimental evidence exists that *gsc* activation and maintenance are regulated differentially because *gsc* is activated shortly after MBT but the expression is not maintained in later stages in embryos that ectopically express *BMP-4*, a gene with ventralizing activity (Fainsod *et al.*, 1994, Hogan *et al.*, 1994). The animal cap experiments presented above, however, show that overexpression of ß-catenin was not sufficient to induce *gsc* expression after MBT.

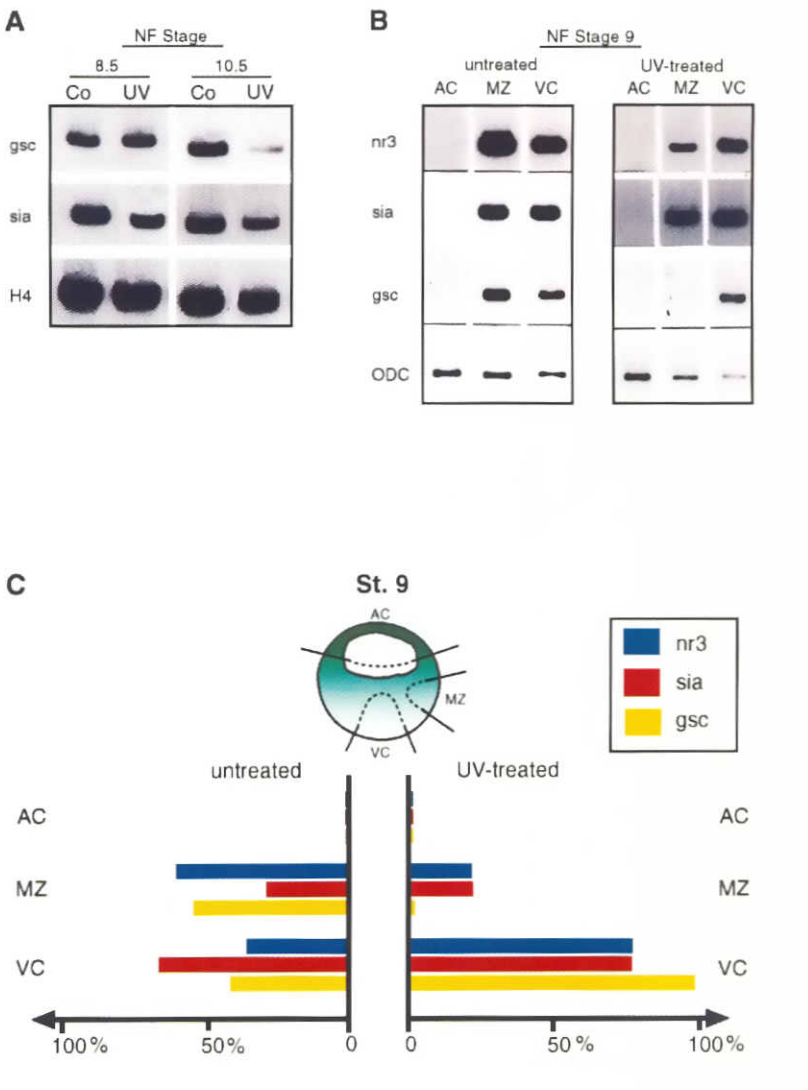

Fig. 2. The expression domains of *gsc*, *nr3* and *sia* are shifted towards the vegetal pole in UV-treated embryos. (**A**) *Total RNA from embryos that were untreated (Co) or treated with UV (DAI 0.46) was analyzed for the expression of sia, gsc and H4 at NF stages 8.5 and 10.5 by RT-PCR.* (**B**) *Embryos were ventralized by UV-irradiation resulting in a DAI of 0.26 judged at stage 30. The embryos used for the experiment were dissected at stage 9 into animal cap (AC), marginal zone (MZ) and vegetal cap (VC). The expression of nr3, sia, gsc and ODC was analyzed by RT-PCR.* (**C**) *The level of transcripts for nr3, sia and gsc were quantified using a Phosphorimager. The distribution profiles of the genes were plotted indicating the percentage of the transcripts present in AC, MZ and VC in untreated and UV-treated embryos. 100% corresponds to the sum of transcripts of the individual genes found in AC, MZ and VC.*

The formation of Spemann's organizer depends on cortical rotation and mesoderm induction

The activation of dorsal-specific genes in the vegetal pole of ventralized embryos is in full agreement with experiments that demonstrated that the dorsal axis-forming activity in 32-cell embryos ventralized by UV-treatment remains at the vegetal pole and does not shift towards the prospective dorsal side (Fujisue *et al.*, 1993). The

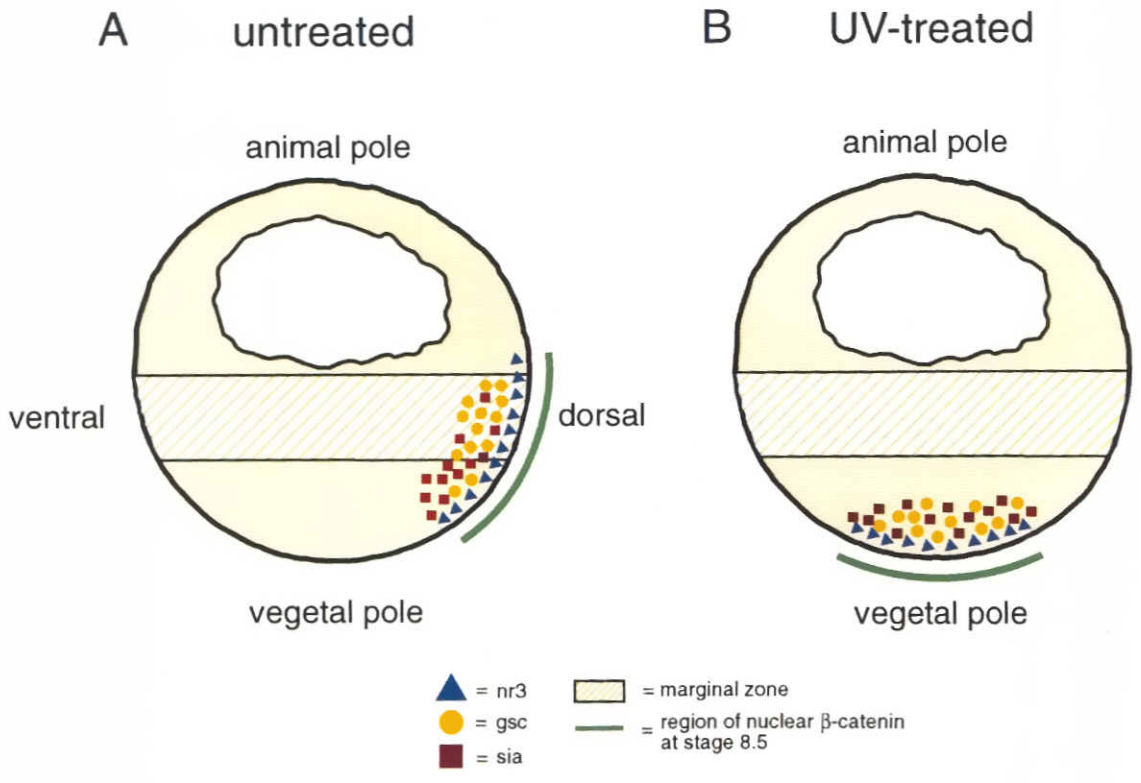

Fig. 3. Axis formation requires cortical rotation and mesoderm induction: a model. (**A**) *In untreated Xenopus embryos, cortical rotation shifts a dorsalizing activity towards the future dorsal side. This process is reflected by the nuclear localization of ß-catenin in the dorsal region. The expression domains of nr3 and sia, which are activated via a process associated with cortical rotation, overlap with the region of the prospective mesoderm in the marginal zone.* (**B**) *In UV-treated embryos, cortical rotation is blocked and nuclear ß-catenin is found in cells at the vegetal pole. Dorsal-specific genes are activated there after MBT but due to the lack of overlap between their expression domain and the mesoderm, the expression of genes such as gsc can not be maintained outside the mesodermal region. Under these conditions, no organizer can form and no axis can be established.*

lack of dorsal axis structures in these UV-treated embryos would then be due to the spatial separation of dorsalizing gene activities and the prospective mesoderm on which they exert their effects (Fig. 3). When the cytoplasmic regions containing the dorsalizing activity at the vegetal pole and the marginal zone are moved such that they overlap, which is achieved by tilting a UV-treated embryo by 90°, axis formation is rescued. Our results show that the formation of a functional and correctly positioned Spemann organizer is dependent on both cortical rotation and mesoderm induction. The importance of an interaction between the egg cortex and the core for the formation of the axis was described many years ago by Dalcq and Pasteels (1937). In their cortical gradient model, the dorsal side forms where the highest value of a cortical factor generated by cortical rotation and a core factor is reached. Our analysis of the spatial activation of genes with dorsalizing activity in UV-treated *Xenopus* embryos as well as recent cortex transplantation experiments by Kageura (1997) revive and strongly support the concept that cortex and core components act synergistically in axis formation.

Experimental Procedures

UV treatment, embryo dissection and microinjection

Fertilized *Xenopus* eggs were irradiated with UV-light (254 nm, 875 mJ) in a Stratalinker (Stratagene) 30 min after fertilization and the embryos remained in the quartz irradiation chamber for 1 h. For the analysis of the RNA distribution, animal caps, marginal zones and vegetal caps were isolated at NF stage 9 (Nieuwkoop and Faber, 1967).

The Dorso-Anterior-Index of the UV-treated embryos was scored after 36 h (Elinson and Kao, 1989). For the ß-catenin overexpression experiments, animal caps were explanted at stage 8 and cultured until control embryos reached stage 8.5 and 10.5. To induce mesoderm in the explants, activin (5U/ml) or bFGF (100 ng/ml) was added to the culture medium. Capped synthetic ß-catenin mRNA was synthesized from psp ß-catenin plasmid linearized with EcoRI using a Message Machine Sp6 Kit (Ambion). Embryos were injected at the 2 or 4-cell stage into the animal region with 300 pg ß-catenin mRNA/embryo.

RNA preparation and RT-PCR analysis

Total RNA from embryos or embryonic fragments was isolated using TRIZOL reagent (Gibco, BRL). Quantitative RT-PCR was performed as described in Niehrs *et al.* (1994). The following primers were used: *gsc*: 5' CTCCCTTACATGAACGTTGGC and 5' TCTGAGATGAACTCTCCTTGC (194bp, 34 Cycles); *nr3*: 5'TGAATCCACTTGTGCAGTTCC and 5'GACAGTCTGTGTTACATGTCC (233bp, 29 cycles) *sia:* 5' CCATGATATTCATCCAACTGTGG and 5' GTTCTCTTCCTAGATCTGGTAC (317bp, 34 cycles); *H4:* 5' AGGGACAACATCCAGGGCATCACC and 5' ATCCATGGCGGTAACGGTCTTCCT (188bp, 20 cycles); *ODC*: 5' GTCAATGATGGAGTGTATGGATC and 5'TCCATTCCGCTCTCCTGAGCAC (385bp, 25 cycles). The annealing temperature for all primers used was 55°C. When no radioactive tracer was used in the PCR reactions, the amplified frag-

ments were separated on 1.5% agarose gels and stained with ethidium bromide (Fig 1A and B). The radioactive PCR products were separated on 7.5% polyacrylamide gels and the gene-specific fragments quantified using a Phosphorimager (FujiX Bas 1000).

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