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Limb and lower-body duplications induced by retinoic acid in mice

(pattern formation/pregastrulation development/developmental abnormalities)

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ABSTRACT **The zygote and subsequent preimplantation stages of early mammalian development are susceptible to certain chemical perturbations that cause abnormal development of the conceptus. In certain cases, disruption in patterns of gene expression could be a primary event leading to abnormal development. To investigate this hypothesis, we treated pregnant mice with *trans*-retinoic acid, a known modulator of gene expression. Treatments were administered at various times during pregastrulation stages and the presumed onset of gastrulation. *trans*-Retinoic acid induced a distinctive set of malformations, as manifest by supernumerary and ectopic limbs and duplication of portions of the lower body, but only when administered during the period of 4.5–5.5 days after mating. (Other malformations were induced at different stages.) The limb and lower-body duplications suggest that exogenous *trans*-retinoic acid may influence not only the pattern for the hindlimbs but also that for the entire lower body. Since it appears likely that the embryos were affected in the late blastocyst and proamniotic-embryo stages, the provocative possibility arises that aspects of pattern formation of limbs and lower body actually occur prior to gastrulation.**

Exogenous *trans*-retinoic acid (RA) has profound effects during embryogenesis, especially on developing and regenerating limbs. Systemic administration of RA in mammals, amphibians, and chickens during limb-bud development generally results in limbs that are attenuated to various degrees at different proximal–distal levels (1). In contrast, the same report also stated that RA administered locally in developing or regenerating limbs of chickens and amphibians leads to pattern duplication as expressed by induction of limb duplication and supernumerary digits.

Recently, a most remarkable effect of RA was observed in the tadpoles of an Indian frog species, *Uperodon systoma* (2). Tadpoles whose tails had been amputated generated hindlimbs instead of tails when placed in water with retinyl palmitate. This phenomenon has also been demonstrated in *Rana temporaria* (3). It was hypothesized that homeotic transformation of tails into legs is initiated by activation, inhibition, or increase in transcriptional activity of certain genes, some of which are responsible for the respecification of tail cells to leg cells (3). A broader hypothesis for this homeotic transformation proposes that the tail blastema are converted by RA to a state that is synonymous to that of the pre-limb-bud flank cells. This respecification is then followed by interaction between the blastema and the stump cells to generate hindlimb sites on the tail (1).

Pattern duplication had not been a feature of RA-induced developmental abnormalities in mice. We report here limb and caudal effects of RA in mice that, up till now, had been induced only in lower vertebrates—i.e., supernumerary limbs in chickens and duplication of the lower portion of the body in amphibians. Unlike in chickens and amphibians, in which RA was applied locally to developing or regenerating limbs or to amputated tails, in mice, RA injections of pregnant females 4.5–5.5 days after mating were found to be effective in inducing the duplication effects. During this treatment window, embryos are in the blastocyst and pregastrulation stages.

MATERIALS AND METHODS

(C3H/Rl × C57BL10/Rl)F_1 females (about 12 weeks old) were mated the morning after ovulation for 30 min to males of the same F_1 hybrid stock and then treated intraperitoneally with RA (Hoffmann–La Roche) at one of the following intervals after mating: 1 h, 25 h, 2 days, 3 days, 4 days, 4.5 days, 5 days, 5.5 days, 6 days, and 7 days. Because of the increasing effects of RA with advancing gestation on periimplantation mortality of the embryos, the dose was varied at different treatment times (see Table 1). The dose was adjusted by an injected volume of solution, with the maximum volume being 0.1 ml. Females were sacrificed on gestational day 18, the uterine contents were scored, and living fetuses were examined for external anomalies. A subset of day 18 fetuses were cleared and stained with alizarin red (bone stains red) and alcian blue (cartilage stains blue). Selected malformations were examined histologically. To stage embryos between gestational days 4.5 and 6.0, unimplanted live embryos were flushed from the uterus, or implanted embryos were histologically sectioned at the implantation site.

RESULTS

Embryonic Lethality. Embryos less than 4 days after mating were relatively resistant to the lethal effects of RA. Treatment of females at 1, 25, 48, or 73 h with 200–300 mg of RA per kg produced only marginal effects on embryonic survival (4–15% lethality). The sensitivity of embryos increased rapidly with gestational age during the period of 4–7 days after mating (Table 1). Treatment at 4 days with 200 mg of RA per kg produced a substantial increase in embryonic lethality, and by day 7, a dose as low as 12.5 mg/kg killed 75% of the embryos. Embryonic death was represented primarily by resorption bodies in all treatment intervals.

Fetal Malformations. Among surviving fetuses, varied external malformations were observed including neural tube, abdominal wall, and head and facial defects (Table 1). The main finding of this study, however, was the induction by RA

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Abbreviation: RA, *trans*-retinoic acid.

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Table 1. Developmental consequences of early gestational treatment with RA

Parameter	Day 4	Day 4.5	Day 5	Day 5.5	Day 6	Day 7	Control
Dose(s), mg/kg	200	50, 75, 100	50, 75	37.5, 50	18.5, 25, 37.5	12.5, 18.5	DMSO
Average dose, mg/kg	200	68	53	41	21.5	15	
Pregnant females, no.	25	81	35	48	60	39	86
Postimplantation deaths, %*	34	25	41	44	41	79	3
Living fetuses, no.	174	706	238	154	299	88	951
Fetuses with defects, %†	7	10	26	18	20	75	0.1
Fetuses with specific malformations, no.‡							
Caudal and limb duplications§	0	23	35	12	0	0	0
Exencephaly	3	8	18	9	60	8	1
Spina bifida	0	0	5	1	0	0	0
Eye defects	9	6	1	0	0	58	0
Abdominal-wall defects	1	12	11	0	3	0	0
Facial anomalies	0	0	0	0	2	19	0
Other anomalies	0	6	3	1	0	0	0

Control is pooled from all experiments. Each female received 0.1 ml of dimethyl sulfoxide (DMSO). Data are shown for the indicated treatment days after mating. Individual doses are those that induced a significant increase in the number of conceptuses affected (deaths plus malformations). Average doses were weighted for the number of surviving fetuses at each individual dose.

*All treatments induced embryonic death diagnosed primarily as resorption bodies. None of the treatments significantly affected the number of implants per female.

†Fetuses with external malformations as a percent of living fetuses. All treatments in days 4.5–5.5 after mating induced caudal/limb malformations.

‡Some fetuses had more than one malformation. All defects were identified by external examination.

§Seventy-three percent of caudal masses bore one or two externally recognizable limbs or rudimentary limb structures. On day 4.5, 7 of 23 and, on day 5, 11 of 35 had only duplicated limbs.

of limb and lower-body duplications exclusively during the period from 4.5 to 5.5 days after mating. The dose of 200 mg/kg used at day 4 and the average dose of 21.5 mg/kg used at day 6 induced substantial levels of embryonic lethality and increased the incidence of fetal malformations; however, these malformations did not include limb and lower-body duplications. The possibility that the duplications were selected out at relatively high doses was ruled out by the results of experiments at day 6 (using a dose of 12.5 mg of RA per kg) and at day 5.5 (using doses of 12.5 and 25 mg of RA per kg), which indicated both a lack of induction of embryonic lethality (data not shown) and a lack of fetal malformations (day 6, 187

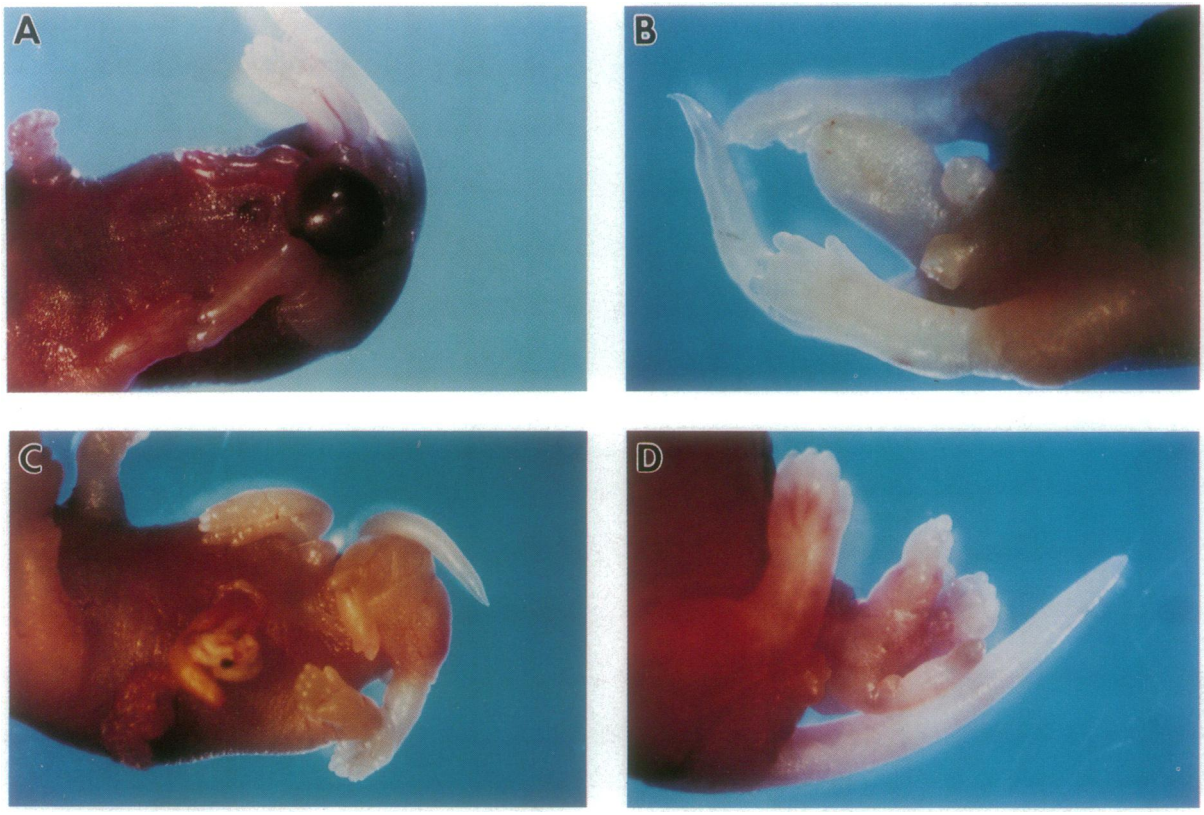

FIG. 1. Limb and lower-body duplications in gestational day 18 fetuses. (*A*) A caudal mass with single projecting rudimentary limb. Additional body and soft-tissue projections were present in some animals. (*B*) Caudal mass composed of soft tissue, without limb formation. The genital tubercles are duplicated. (*C*) A caudal mass with two well-formed limbs is suggestive of a duplicated lower-body portion. The digits are normal in one of the limbs and attenuated in the other. Other similar fetuses have two well-formed limbs on the caudal mass. (*D*) A single hindlimb is duplicated and has additional unidentifiable tissue projection. The caudal region is unaffected.

fetuses examined; day 5.5, 159 and 121 fetuses examined, respectively).

The unique limb and caudal malformations exhibited a range of phenotypic expressions (Figs. 1 and 2). The extreme showed a caudal mass that duplicated a portion of the torso. It supported an extra set of hindlimbs and, often, a tail-like structure. Generally, an extra genital tubercle was also present. The extra set of limbs ranged from being fully formed to being severely attenuated. In cases where the orientation was clear, the pair of limbs consisted of one left and one right and was attached to a reduced pelvis (Fig. 2*B*). While the extra hindlimbs were often of reduced size, the primary ones were almost always normal. An intermediate expression of the defect consisted of the presence of a caudal bulbous mass, rostral or caudal to the genital tubercle, from which a generally solitary structure projected. This structure varied from an amorphous soft-tissue nodule to a limb of variable size (occasionally normal) and deformity (Fig. 1 *A* and *B*). An extra genital tubercle was usually associated with the caudal mass. Some caudal masses without an externally recognizable limb contained rudimentary limb structures, but these bones could not be consistently categorized (Fig. 2*C*). It is likely that the intermediate to extreme manifestations of the caudal malformations, in fact, represent variations along a phenotypic spectrum of duplications of a pattern for the caudal portion of the body. These malformations were not associated with bifurcation of the vertebral column, and thus, they do not appear to involve partial or complete duplication of the primary body axis. Sections of caudal masses from 10 fetuses revealed organized proliferation of mainly mesenchymal tissues, occasional ectopic mature neural tissue, and in one case an ectopic kidney. The mild form of the defect, which was generally unilateral, involved duplication of all or only a portion of one hindlimb without caudal mass formation (Figs. 1*D* and 2*D*). Occasionally, projections that are unidentifiable as tail or toe were found on the duplicated limbs.

In contrast to the numerous supernumerary and duplicated hindlimbs, only one possible duplication involving the forelimb was observed (Fig. 3). In this case the ectopic limb was in the correct general location for a forelimb, but it was not well enough formed to be definitively classified as such.

Other types of malformations were also induced during the window from days 4.5 to 5.5 and at preceding and subsequent intervals (Table 1). Exencephaly and eye and abdominal-wall defects were the predominant malformations induced by RA treatments administered at day 4 after mating or earlier (data not shown). For RA treatments between days 4.5 and 6 after mating, the incidences of abdominal-wall and eye defects decreased and exencephaly generally increased. By day 7, the anomalies observed were of an entirely different spectrum with eye and unique facial anomalies predominating. Increasing the dose of RA produced increases in the number of total malformations, caudal masses, limb duplications, and dead implants; however, the spectrum of anomalies was not affected by varying the dose. The types of anomalies induced at 1 h and at 1, 2, or 3 days after mating (data not shown) are generally similar to those induced at 4 days (Table 1).

Staging of Embryos. The limb and lower-body duplications were induced by RA only during the period from 4.5 to 5.5 days after mating, which is generally assumed to be prior to the onset of gastrulation. Because of the general understanding that pattern specification occurs during gastrulation, we confirmed this stage specificity by analyzing embryos from untreated female mice. Staging of randomly selected embryos from a total of 36 untreated litters revealed that on day 4.5 after mating, 46% (53 of 115) of blastocysts had not yet

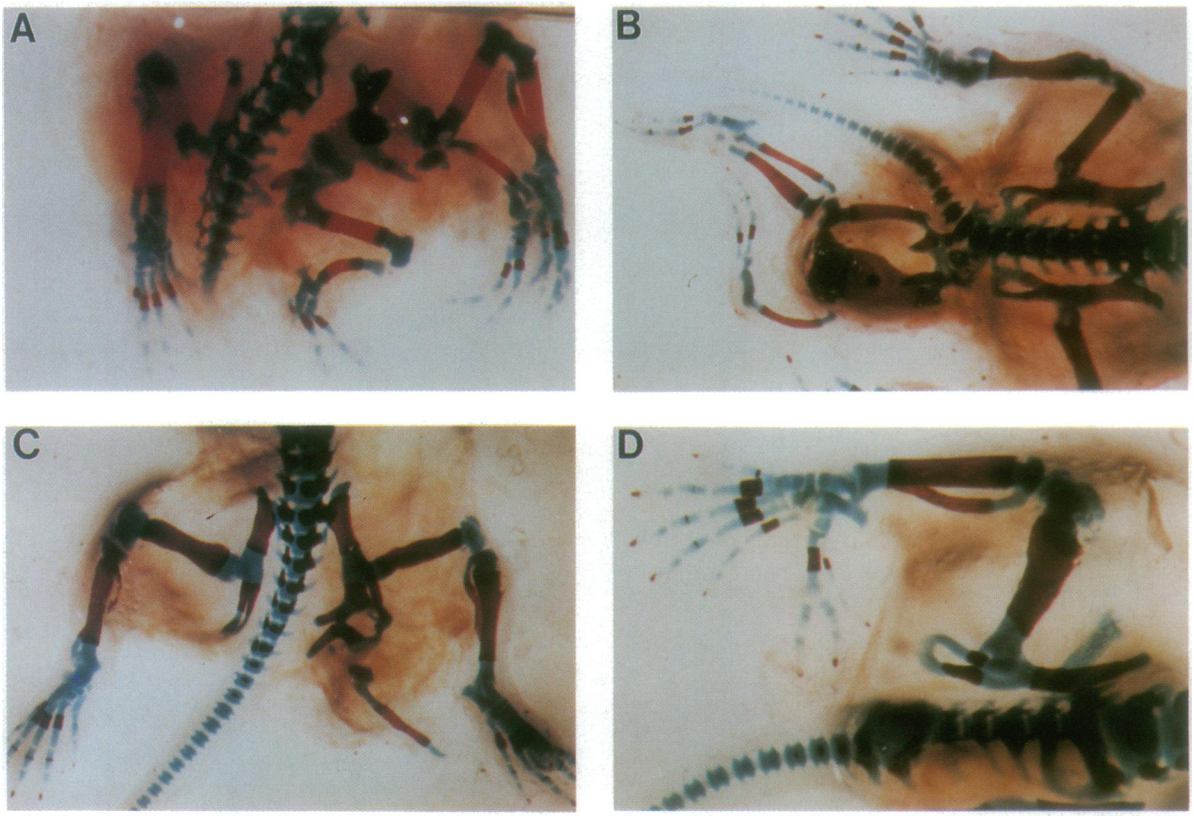

FIG. 2. Skeletal manifestations of caudal and hindlimb malformations in gestational day 18 fetuses. (*A*) The fully formed supernumerary hindlimbs are attached to a modified pelvis, which is duplicated only on one side. The vertebral column is normal. (*B*) A caudal mass incorporates a duplicated pelvis and a broadened femur with partially reduced distal limb formation. (*C*) Some caudal masses without externally recognized limbs contained rudimentary limbs unrecognizable as specific bones. (*D*) Duplication of a distal paw, with normal proximal limb, is part of a spectrum of limb-confined duplications.

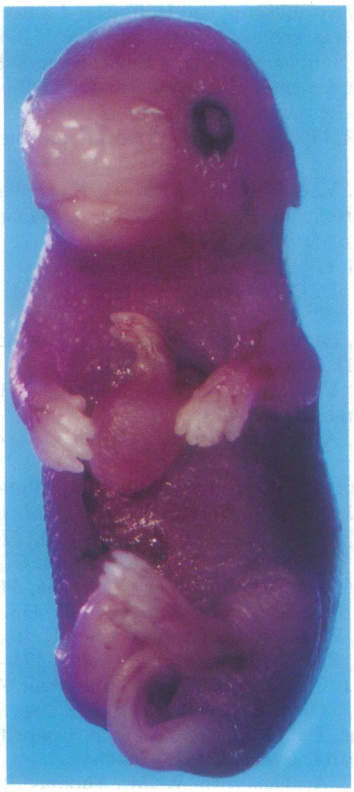

FIG. 3. RA-induced thoracic ectopic limb development in gestational day 18 fetus.

implanted; on day 5.0, implantation was complete; on day 5.5, 64% (16 out of a random sample of 25 embryos) had proamniotic cavities with the rest still uncavitated; and on day 6.0, all (a random sample of 26) egg cylinders were fully cavitated but none had any sign of primitive-streak formation, indicating that gastrulation had not yet begun.

DISCUSSION

The limb and lower-body malformations described here are unique among those induced by chemicals and ionizing radiation. In a recent review (4) of mammalian studies investigating drug-induced limb defects, none of 171 studies, including those that used RA as the teratogen, reported the observation of similar phenotypes. It appears that the reason earlier studies failed to uncover this developmental phenomenon is that in the vast majority of these studies, gravid females were treated with the teratogen in question only during gestational stages when the embryos were expected or known to be gastrulating (beginning 6.5 days after mating) or were already in the state of organogenesis. The single-pulse RA treatments in the present study, on the other hand, were given to females that were in earlier gestational stages than those in most prior teratogenesis studies. The same phenomenon of limb and lower-body duplications has, subsequently, been observed also in CD-1 mice (a similar experiment was conducted at the National Institute of Environmental Health Sciences by J.B.B., unpublished data). RA is a human teratogen producing craniofacial anomalies, microcephaly, hydrocephaly, conotruncal malformations of the heart, absence of thymus, and, only rarely, limb abnormalities that are exclusively of the reduction types similar to those observed in animal studies (5).

The lower-body and hindlimb duplications induced in embryos of treated pregnant mice have analogies with other findings. They resemble, in principle, those generated in amputated tails of the frog, *Rana temporaria* (3). There is, however, a distinct difference: in the mouse, extra limbs were often reduced and the primary limbs were normal, whereas the reverse was true in the frog. Considering the effects of systemic administration on limb-bud development (1), this might be because limb generation in amputated tails of frogs occurred only when RA treatment coincided with hindlimb-bud development. In the mouse, on the other hand, the treatment time preceded the time of limb-bud development by several days.

The spectrum of malformations induced by RA on days 4.5–5.5 resembles that observed in mice carrying the dominant mutation, disorganization (*Ds*). This mutation causes ectopic duplications of limbs, paws, and girdles, and supernumerary organoids and tissues (6). Human counterparts of this mutation have also been reported (7, 8).

The hindlimb and lower-body duplications that are so strikingly similar in the mouse and the frog may involve the initiation of separate patterns. For limb duplication in amputated tadpole tails, it was proposed that RA converts the pattern-formation-competent tail blastema cells to a positional value that is posterior-ventral-proximal with respect to the limb (1). In other words, it is suggested that RA specifies a whole limb field. Since the hindlimbs in the *Rana temporaria* study tended to be in pairs associated with pelvic girdles, it was thought that RA may actually be inducing a pair of limb fields that can be the precursors for additional fields (3). However, in the mouse there were rare cases in which both the limb and lower-body duplications were present in the same fetus. Furthermore, lower-body duplications were often associated with their own tail-like structure, genital tubercle, and mesenchymal and neural proliferations. In frog, notochord was observed with the duplications (3). This suggested that, instead of a pair of limb fields, there may have been initiation of a pattern for the whole lower body. Thus, in vertebrates, exogenous RA may be specifying not only the pattern for the hindlimbs but also the pattern for the entire lower body. The genes that are suspected to have a role in RA-induced limb pattern specification have been reviewed extensively by others including two recent reviews cited in this report (1, 3).

The targets for RA in amputated tadpole tails appear to be the blastema cells. In the mouse, we are confronted with a much more complex situation. We may assume that RA specifies the duplicated limb and lower-body patterns in pattern-formation-competent target cells. The window of induction is 4.5–5.5 days after mating when embryos are clearly in the pregastrulation stages (i.e., blastocyst and preamniotic embryo). If RA, and not its metabolites, is responsible for the duplications, then the target cells have to be present in the pregastrulation stages. Alternatively, the pattern-formation-competent cells may exist during early gastrulation; in which case, the duplicated patterns would have been initiated by a persistent RA metabolite. It has been hypothesized that the effects of RA in modifying positional specification in *Xenopus* occur, at least partially, by metabolism to 4-oxo-retinoic acid (9). A study in which the critical periods for RA treatments later in development were compared with the periods for embryonic events assumed to be associated with various malformations showed exact coincidence in some and delays of up to about 1.5 days in others (10).

The initiating event for the duplicated patterns occurs only during a narrow window in early embryonic development and, regardless of the stage at which pattern specifications take place, the question arises as to whether the window coincides with that for normal patterning. Whether pattern-formation-competent cells exist prior to or during gastrulation cannot at present be determined and confirmatory studies to resolve the issue are not likely to be straightforward.

Nevertheless, this provocative issue deserves to be studied systematically because the possibility that pattern-formation-competent cells exist during prestreak stages challenges the predominant view that pattern specification occurs during gastrulation. Finally, the similarity between the present results and those found recently with two frog species (2, 3) provides an extraordinary opportunity to study the genetic, molecular, and embryological nature of RA-induced pattern formation in vertebrates.

This study was, in fact, designed to follow up on the hypothesis that disruption in the pattern of embryonic gene expression during preimplantation stages can lead to fetal malformations (11, 12). While the limb and lower-body duplications highlight the developmental effects of RA in this report, the induction of other types of external malformations—e.g., abdominal wall, facial, and eye defects and exencephaly—is also of considerable significance. There are indications that the type of malformations induced by RA in early gestation is stage-dependent (Table 1). Agents besides RA have also been found to induce eye and abdominal-wall defects when females were treated during very early stages of gestation, including that when their conceptuses were merely zygotes (12). Because of the increasing importance of pregastrulation stages in developmental toxicology (13, 14), additional studies of the spectrum and association of developmental anomalies induced by RA during days 4–7 of gestation will be valuable, especially if these include examination of the internal organs and of the skeletal system.

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