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COMMENTARY

How Knockout Mouse Lines Will Be Used to Study the Role of Drug-Metabolizing Enzymes and Their Receptors during Reproduction and Development, and in Environmental Toxicity, Cancer, and Oxidative Stress

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ABSTRACT. The dioxin-inducible mouse [*Ah*] battery contains at least six genes that "cross-talk" with one another and are believed to play important roles in reproduction and development, and in environmental toxicity, cancer, and oxidative stress. In addition to two P450 genes, *Cyp1a1* and *Cyp1a2*, this laboratory has shown that the four Phase II [*Ah*] genes include: NAD(P)H:menadione oxidoreductase (*Nmo1*); a cytosolic "class 3" aldehyde dehydrogenase (*Ahd4*); a UDP glucuronosyltransferase having 4-methylumbelliferone as substrate (*Ugt1a6*); and a glutathione transferase having 2,4-dinitro-1-chlorobenzene as substrate (*Gsta1*, Ya). The Ah receptor-mediated coordinate induction is controlled positively in all six [*Ah*] battery genes. Oxidative stress up-regulates the four Phase II [*Ah*] genes. This laboratory is generating conventional, plus inducible, knockout mouse lines having homozygous disruptions in the above-mentioned genes; this novel methodology is described herein. If the conventional knockout is healthy and viable, the mouse line would be useful for studies involving environmental agents. If the conventional knockout is lethal during development, this model would be important for developmental biology, but the inducible (also called conditional) knockout can still be used—at selected ages and even in selected tissue or cell types—for studies designed to understand the mechanisms involved in reproduction and development, and in environmental toxicity, cancer, and oxidative stress. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:249–254, 1997.

KEY WORDS. [*Ah*] gene battery; mouse genetics; Cre recombinase; *loxP* sites; dioxin; polycyclic aromatic hydrocarbons; drug-metabolizing enzymes; Ah receptor

The explosion of molecular biology and recombinant DNA technologies began in the late 1970s. In the past several years, these advances, which have occurred at increasingly unbelievable rates, are now leading to the generation of conventional plus inducible "knockout" mouse lines in which one can characterize the phenotype of intact animals–*in utero* or later in life—carrying the homozygous disruption of the gene of your choice. At the beginning of these studies 3–6 years ago, it was expected that knocking out a gene in a critical signal transduction pathway would probably be lethal to the animal, thereby aiding studies of developmental biology in particular. To everyone's astonishment, few knockout mouse lines are embryonically lethal, underscoring the biologic and evolutionary importance of redundancy of the components in almost every signal transduction pathway. There are, however, examples of homozygous lethality before birth [1, 2; reviewed in Ref. 3] and examples of heterozygous lethality *in utero* [4, 5].

This laboratory has studied for more than 20 years the dioxin-inducible mouse [*Ah*] gene battery. This battery comprises at least six genes that "cross-talk" with one another; these genes are believed to play important roles in reproduction and development, and in environmental toxicity, cancer, and oxidative stress [discussed in detail in Refs. 6–8]. In addition to two P450 genes, *Cyp1a1*[‖] and

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‖ *Abbreviations:* *Cyp1a1* and CYP1A1, mouse cytochrome P450 1A1 gene and enzyme; *Cyp1a2* and CYP1A2, mouse cytochrome P450 1A2 gene and enzyme; *Nmo1* and NMO1, mouse NAD(P)H:menadione oxidoreductase [also called quinone reductase, DT-diaphorase] gene and enzyme; *Ahd4* and AHD4, mouse aldehyde dehydrogenase-3c gene and enzyme; *Ugt1a6* and UGT1A6, mouse UDP glucuronosyltransferase-1A6 gene and enzyme; *Gsta1* and GSTA1, glutathione transferase (Ya or class α) gene and enzyme; AHR, Ah receptor; ES, embryonic stem; HAT, medium containing hypoxanthine, aminopterin, and thymidine; *neo*, neomycin phosphotransferase gene conferring resistance to G418; HSV-*tk*, herpes simplex

Cyp1a2, we have shown that four Phase II [*Ah*] genes include: *Nmo1*, *Ahd4*, *Ugt1a6* and *Gsta1*. AHR-mediated coordinate induction is controlled positively in all six [*Ah*] battery genes. Oxidative stress up-regulates all four Phase II [*Ah*] genes, and binding of the AHR to the electrophile response element appears to participate in this process [9].

This laboratory is currently generating conventional, plus inducible, knockout mouse lines having homozygous disruptions in the above-mentioned genes/pathways; below we describe this novel methodology. If the "conventional" knockout is viable and fertile, this mouse line will be useful for studies of environmental toxicity or carcinogenesis. If the conventional knockout is lethal during development, however, this model will provide important information about this gene during embryogenesis or fetal development. Yet, the "inducible" knockout can still be used—at selected ages and even in selected tissues or cell types—for studies involving the mechanisms of environmental toxicity and cancer. Such inducible knockout lines may elucidate for the first time the interplay between tissues or organs, e.g. aberrant liver and immune system development in *Ahr(−/−)* mice [10], and may also help us to understand secondary effects of a disease process that have little or nothing to do with the disrupted gene, e.g. multifocal inflammation in TGFβ1(−/−) mice [11]. Inducible knockouts, thus, can circumvent these secondary effects and reveal truly primary effects.

WHAT IS Cre RECOMBINASE AND loxP?

The Cre recombinase and *loxP* are an enzyme and a DNA recognition site, respectively, used by the bacteriophage P1 [12]. The *loxP* site represents 34 bases: an inverted repeat of 13 bp, plus the 8-bp spacer sequence that imposes directionality (same orientation = excision; opposite orientation = inversion) on the recombinant event. It was discovered that two *loxP* sites—as far apart as 200 kb [13]—will be recognized by the Cre recombinase, which will then excise all the DNA between these two *loxP* sites (Fig. 1, top). Intriguingly, Cre recombinase has even been shown to mediate recombination and excision between nonhomologous chromosomes [14].

How to Introduce the loxP *Sites*

Site-specific recombination using the Cre-*loxP* system has been shown to work in both mouse ES cells [15; reviewed in Ref. 16] and in transgenic mice [17, 18; reviewed in Refs. 19 and 20]. The two-step strategy is illustrated in the bottom panel of Fig. 1. In cultured ES cells, one can generate

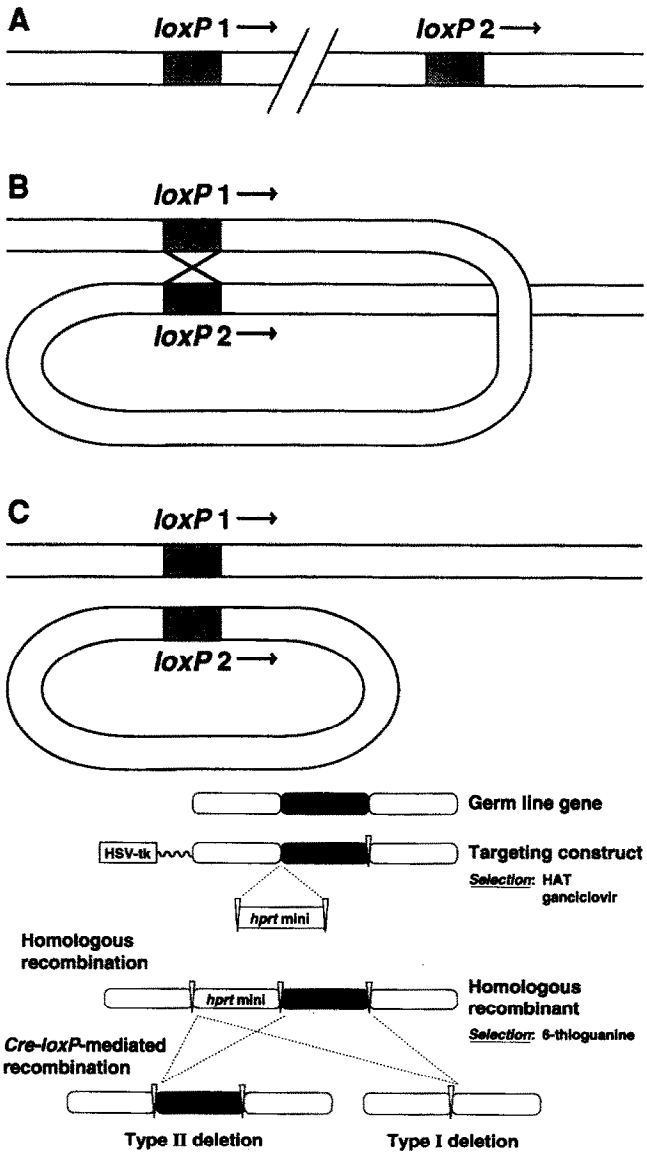

FIG. 1. Top: Diagram of *loxP* sites inserted in the same orientation some distance apart on the DNA molecule (A); action of Cre recombinase as it aligns these two sites (B) and excises all the DNA between these two sites (C). Bottom: Strategy for generating a gene segment (closed bar) flanked by *loxP* sites in ES cells [12]. Shown are: the germline gene, a corresponding targeting construct generated *in vitro*, the resulting homologous recombinant in ES cells, and the two types of *Cre-loxP*-mediated deletions that will be isolated. The *loxP* sequences are represented by thin triangles. The position of the selection marker cassette containing the *hprt* mini-gene is indicated.

in parallel (a) the *loxP*-flanked ("floxed") gene (or gene segment), and (b) a deletion of the same piece of DNA.

In the first step, three *loxP* sites—in addition, such selection marker genes as *neo*, *HSV-tk* or *hprt* [21, 22; reviewed in Ref. 23]—are introduced by way of homologous recombination into (presumably) nonfunctional regions of the target gene. There are reports of one selection marker being preferred over another. For example, the HSV-1/*tk* gene is being used less, because of possibly causing male

virus thymidine kinase gene conferring sensitivity to ganciclovir; *hprt*, hypoxanthine phosphoribosyltransferase gene conferring resistance to HAT medium and sensitivity to 6-thioguanine; *Cre* and CRE, bacteriophage P1 gene and enzyme; and "floxed," insertion of *loxP* sites to flank a gene or gene segment that Cre recombinase can then act upon.

sterility in some transgenic lines [24]. Typically, an intron, exonic nontranslated segment, or 5′- or 3′-flanking region of the gene is used for the locations to insert the *loxP* sites. Ofttimes, the HSV-*tk* gene is placed at one end of the construct for counter-selection against cells in which the vector has integrated randomly. Then the targeting construct (Fig. 1, bottom) is electroporated into *hprt*$^-$ ES cells, and the transfected cells are grown on a mitomycin C-treated mouse embryo fibroblast feeder layer. Later, individual HAT-resistant ganciclovir-sensitive clones are isolated. Finally, polymerase chain reaction and Southern blot hybridization analyses are used to confirm that homologous recombination has succeeded within the gene in the targeted ES cells and that the targeted *hprt* mini-gene and the single distal *loxP* site have been transferred successfully into ES cell lines (homologous recombinant, Fig. 1, bottom).

In the second step, the *Cre* gene encoding the CRE enzyme is expressed in the genetically modified ES cells, via transient transfection or injected with small amounts of protein. Three different deletions are possible. The Type I deletion (Fig. 1, bottom) results in excision of the target gene from the genome of the ES cells, i.e. this will become the conventional knockout. The Type II deletion, on the other hand, results in a floxed gene at the targeted locus. This will become the inducible knockout. The third possibility, which deletes the target gene but leaves the *hprt* gene in the genome, will not be observed because ES cells carrying such a deletion will die after 6-thioguanine treatment during selection of the Type I and Type II deletion mutants.

GENERATION OF TRANSGENIC MOUSE LINES CARRYING THE TYPE I AND TYPE II TARGETED ALLELES

The protocol for the generation of chimeric mice—in which the coat color phenotypic is used for selection—is well established [25]. Briefly described, targeted ES cells (derived from 129/SV mice, *agouti*) are injected into the blastocoele cavity of 3.5-day embryos bearing the *nonagouti* phenotype. The resulting chimeric blastocysts are then transferred to the uterus of a pseudopregnant female. Identification of chimeric pups can be determined by the presence of the agouti coat color at 7–10 days of age. After subsequent crosses, a completely agouti coat color usually, but not always [discussed in Ref. 26], denotes that there has been germline transmission by the father.

Generation of the Conventional Knockout Mouse Line

Mouse lines homozygous for the Type I (conventional knockout) gene disruption can then be generated by breeding the F_1 heterozygotes described above. If the knockout is viable and fertile (e.g. as this laboratory found for its *Cyp1a2(−/−)* mouse [26], this line should be valuable for drug metabolism, environmental toxicity, and cancer studies. If the conventional knockout is nonviable, with death occurring at some gestational age, this line can suggest an important role of the homozygously disrupted gene during that stage of embryogenesis; however, death *in utero* would not provide the researcher with a suitable model system for studying environmental toxicology, carcinogenesis, or oxidative stress. If the conventional knockout exhibits very poor viability and fertility, e.g. the *Ahr(−/−)* mouse [10], such a line also might not be convenient for definitive toxicity or cancer studies.

Generation of the Inducible, or "Conditional," Knockout Mouse Line

This mouse is typically produced from two mouse lines. The Type I heterozygote, described above, is bred to the Type II heterozygote, to give progeny that carry one allele as a deletion and the second allele as the floxed gene. Such a heterozygous mouse will (a) require less Cre recombinase needed for excision, and (b) decrease the chance of translocation (and other unanticipated events) that may alter or scramble the chromosome. This, then, is the first mouse line.

The second mouse is a transgenic line in which the *Cre* gene is expressed under the control of a tetracycline-responsive promoter. In the original system with the wild-type *tet* repressor [27] or with a fusion tetracycline-controlled *trans*-activator protein (tTA) [28–30], the cell line or animal is kept on tetracycline to keep gene activity from occurring, and removal of tetracycline causes activation of the gene (Fig. 2, top). In a recent modification of this system, termed the "reverse tet system" (Fig. 2, bottom), a transactivator has been developed that reverses the DNA-binding properties, i.e. the *trans*-activator requires the presence of tetracyclines for binding to *tet* operator sequences. One of the more recently developed *trans*-activators [31, 32] fused the activating domain of viral protein VP16 of herpes simplex virus with a mutant *tet* repressor from *Escherichia coli;* this transactivator thus requires tetracycline analogues for specific DNA binding. Doxycycline has now been shown to be the most potent activator [31]. Also, an interferon-responsive promoter has been shown recently to control Cre recombinase expression in the intact mouse [33]; however, one might be wary to use this promoter if the gene being studied is associated with such cellular processes as cytokine production, inflammation, or the acute phase response.

Mouse lines having the *Cre* gene under the control of a minimal promoter (as well as tissue-specific promoters) fused to the *tet* operator sequences, plus the rtTA *trans*-activator, are being produced [12; reviewed in Refs. 19 and 20]. Such mouse lines will undoubtedly be sold commercially very soon. The advantages of these mouse lines would be the possibility of breeding such a mouse with any other mouse line carrying the floxed gene of your choice. Inducible knockout lines specific for CRE activity in the liver, lung, brain, and heart are all expected to be available within the year.

By breeding the mouse carrying the Type I deletion allele

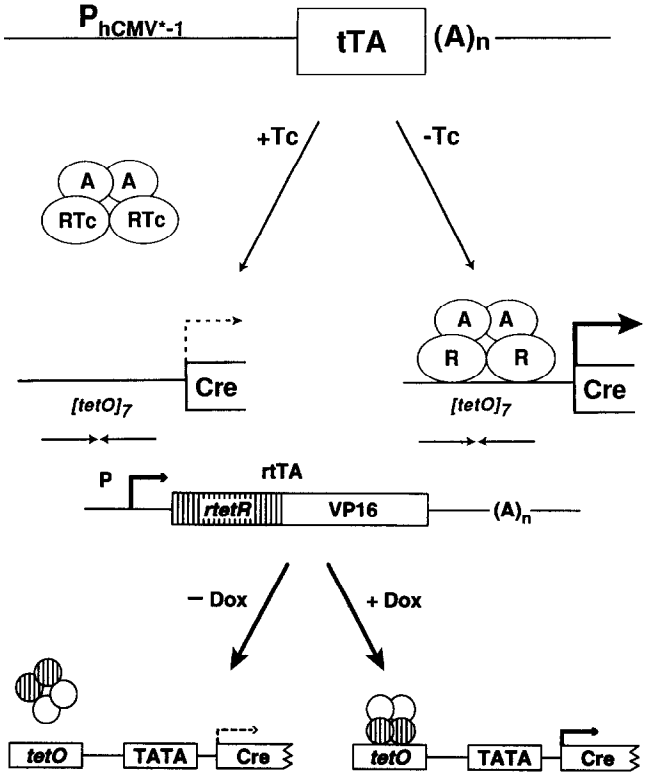

FIG. 2. Top: Diagram of an early system [27] in which a fusion tetracycline-controlled *trans*-activator protein (*t*TA) is followed by a polyadenylation (A_n) site. The minimal promoter-*tet* operator construct consists of seven *tet* operators located upstream of a minimal sequence of the human cytomegalovirus immediate early (CMV IE) promoter. In the presence of tetracycline (Tc), the *trans*-activator (consisting of two A and two RTc units) will not recognize its specific DNA target sequence (*tetO*); thus, transcription of the Cre gene will not occur (dashed arrow). Removal of tetracycline will result in binding of the *trans*-activator to *tetO*, which allows transcriptional activation of the Cre gene (bold arrow). Bottom: Schematic illustration of the "reverse tet system" [31]. The gene encoding rtTA is composed of *rtetR* (the mutant *tet* repressor *R*) and the VP16 activation domain, driven by an appropriate promoter *P* and followed by a polyadenylation (A_n) site. In the absence of the effector doxycycline (–Dox), the *trans*-activator does not recognize its specific DNA target sequence (*tetO*); therefore, transcription of the Cre gene will not occur (dashed arrow). Addition of the effector (+Dox) will result in binding of rtTA to *tetO*, which allows transcriptional activation of the *Cre* gene (bold arrow). The minimal promoter-*tet* operator construct shown here is identical to the tTA-responsive promoter $P_{hCMV*-1}$, which consists of seven *tet* operators located upstream of a minimal sequence of the CMV IE promoter [28]. Tissue- and cell type-specificity of the system is being achieved in numerous laboratories by the selection of suitable tissue- and cell type-specific signals for expression of the *trans*-activator, in place of the $P_{hCMV*-1}$.

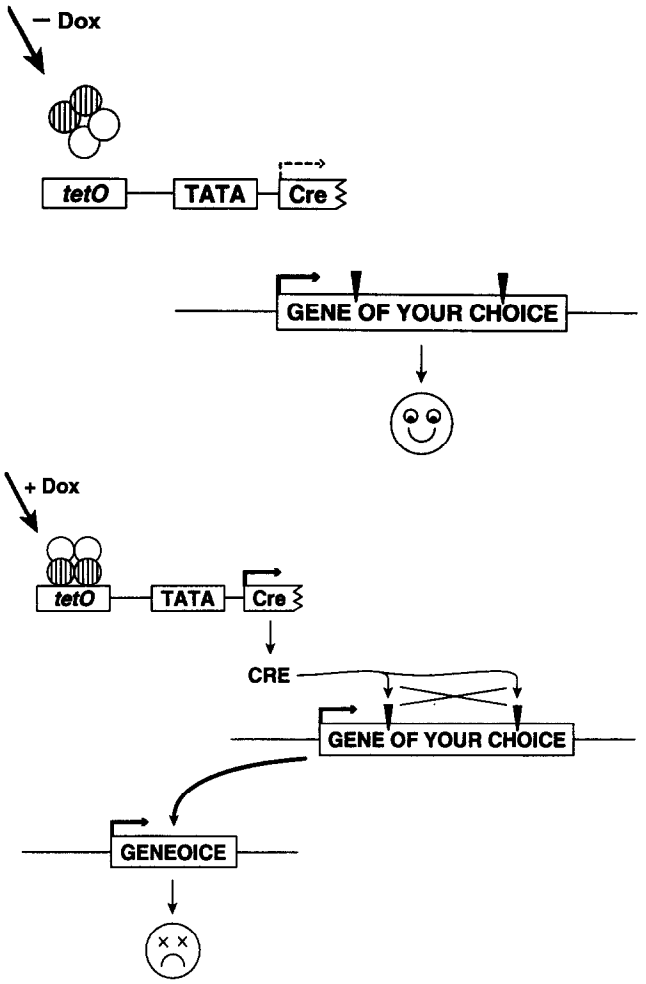

FIG. 3. Top: Diagram of the conditional knockout double-transgenic mouse, having the doxycycline-inducible Cre recombinase gene in place but turned off, and the gene of your choice—floxed yet functioning normally in the intact animal. Bottom: Diagram of the conditional knockout double-transgenic mouse following doxycycline treatment. The floxed segment of the "gene of your choice" has been excised, rendering the gene nonfunctional. Disruption of the floxed gene can even be reversible, just by removing doxycyline treatment.

and the Type II (floxed) targeted allele with the mouse containing the inducible *Cre*, one now has one copy of the "gene of your choice" (Fig. 3, top) functioning properly in the intact animal—yet awaiting the signal for disruption. Oral or subcutaneous treatment of the mouse with doxycycline (Fig. 3, bottom) then turns on the *Cre* gene, thereby disrupting the "gene of your choice" at a specified age. Hence, one could study the function of the gene of your choice at, for example, gestational day 15, 6 weeks post partum, or 10 months of age. And, the conditional gene knockout can be induced globally (in all tissues of the mouse) or in selected tissue or cell types, dependent on tissue- and cell type-specific promoters currently being designed and studied.

OTHER INDUCIBLE KNOCKOUT CANDIDATE PROMOTERS

The doxycyclin-responsive bacterial promoter has been shown recently in mice to control regulation of expression of an individual gene over five orders of magnitude [34; modified in Ref. 31] and is now emerging as the system of

choice. Another recently described successful system uses the insect ecdysone-inducible promoter [35]. These inducible systems do not suffer from the problems of: (a) an interfering inducer (such as heavy-metal ions, steroid hormones, dioxin, or heat shock) that evokes its own pleiotropic effects that might complicate the analysis of the resulting phenotype, or (b) many promoter systems that have high basal levels of activity in the noninduced state, thereby preventing the shut-off of the gene under study and resulting in only modest amounts of fold-induction [reviewed in Refs. 36 and 37].

THE "NEIGHBORHOOD EFFECT"

It has become increasingly appreciated that (a) just where a transgene is inserted, (b) how much of the gene segment is removed, and (c) heterogeneity of the genetic background of the knockout line can all contribute to dramatically different phenotypes [38, 39; reviewed in Refs. 40–42]. It should be appreciated that, for example, a C57BL/6J (from Jackson Laboratory) and a C57BL/6N (from NIH) have diverged from one another for more than 45 years and, therefore, should not be considered genetically identical. This neighborhood effect may explain the major reported differences in phenotype between two *Cyp1a2(−/−)* lines [26, 43] and between two *Ahr(−/−)* lines [10, 44]. Given the same two mouse substrains used (e.g. injecting 129/SV-derived ES cells into the blastocoele cavity of C57BL/6J embryos), the resulting knockout mouse lines will contain some unknown admixture of these two genetic backgrounds, which, of course, will vary from one mouse to the next within the knockout line (just as human siblings would vary in the same family). Each knockout line generated, therefore, must be further bred if one wishes to approach homogeneity of genetic background (and, thus, to achieve a decrease in interindividual variability during any experiment). Given >95% homogeneity of (e.g. C57BL/6J) background still does not guarantee identical phenotypes, if different targeting strategies had been used to remove different numbers of base pairs from the same gene; for example, it is feasible that one but not the other strategy might excise an exon from an unknown activator or inhibitor gene on the antisense DNA strand, somehow affecting the phenotype of the knockout mouse.

CONCLUSIONS

In summary, just as the *Ahr(−/−)* mouse line has been found to exhibit diminished viability and fertility [10], we anticipate that *Cyp1a1(−/−)* and *Nmo(−/−)* conventional knockout lines might be similarly affected. We therefore have begun to generate conventional plus inducible knockout lines for these and other above-mentioned genes that participate in the [Ah] battery and appear to play important roles in reproduction, development, toxicity, cancer, and oxidative stress. Other systems in the planning stages include: (a) insertion of human alleles in place of the disrupted mouse orthologous gene, and (b) generation of tissue- and cell type-specific conventional plus inducible knockout mouse lines.

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