BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **219,** 70–75 (1996)
ARTICLE NO. 0183

Suppression of the Neoplastic Phenotype by Replacement of the *Tsc2* Gene in Eker Rat Renal Carcinoma Cells

Kenji Orimoto,* Haruo Tsuchiya,* Toshiyuki Kobayashi,* Tamotsu Matsuda,† and Okio Hino*,[1]

**Department of Experimental Pathology, Cancer Institute, 1-37-1 Kami-ikebukuro, Toshima-ku, Tokyo 170, Japan; and †Department of 3rd. Internal Medicine, School of Medicine, Kanazawa University, Kanazawa 920, Japan*

Received December 18, 1995

The hereditary renal carcinoma (RC) in the rat, originally reported by R. Eker in 1954, is an excellent example of a Mendelian dominantly inherited predisposition to a specific cancer in an experimental animal. Recently, we have identified a germline mutation of the tuberous sclerosis (*Tsc2*) gene in the Eker rat (*Nature Genetics* **9,** 70–74, 1995), suggested to be a novel tumor suppressor gene, fitting Knudson's two-hit hypothesis. In this study, the effect of wild-type *Tsc2* gene expression in Eker RC cells was tested using a tetracycline-responsive promoter system. Transfection and expression of an exogenous *Tsc2* gene affected both cell morphology and growth rate. This demonstration of suppression of the neoplastic phenotype provides direct evidence for an essential role of the *Tsc2* gene in tumorigenesis.

The Eker rat hereditary renal carcinoma (RC) is an excellent example of a Mendelian dominant predisposition to a specific cancer in an experimental animal (1). We have reported that a germline insertion in the rat homologue of the human tuberous sclerosis gene (*TSC2*) gives rise to the dominantly inherited cancer in the Eker rat model (2,3). We have recently described the entire cDNA and genomic structure of the rat *Tsc2* gene (4). The deduced amino acid sequence (1743 amino acids) shows 92% identity to the human counterpart. Surprisingly, there are a great many coding exons (41 in total) with small sized introns spanning only ~35 kb of genomic DNA. Two alternative splicing events [involving exons 25 (129 bp) and 31 (69 bp)] make for a complex diversity of the Tsc2 product. The detection of loss of heterozygosity (LOH) of the wild-type allele even in the earliest preneoplastic lesions, e.g., phenotypically altered renal tubules, supports the hypothesis that a second somatic mutation (second hit) might be a rate-limiting step for renal carcinogenesis in the Eker rat model of dominantly inherited cancer, as well as providing evidence of a tumor suppressor nature for *Tsc2* gene function (5, 6).

The function of the *TSC2* gene product (called 'tuberin' in the human case) is not yet understood, although it contains a short amino acid sequence homology to ras family GTPase activating proteins (Rap1 GAP) (4, 7, 8). It is therefore important to establish an expression system in mammalian cells to study the function of the *Tsc2* family gene products. In the case of tumor suppressor genes, e.g., the *p53* gene, it is reportedly difficult to establish stable reconstituted clones due to toxicity to the host cells. Therefore, in the present study, we used the tetracycline-regulated transactivator and operator plasmids developed by Gossen and Bujard (9). Regulated expression of wild-type *Tsc2* in Eker rat renal carcinoma resulted in growth inhibition and alteration in morphological properties.

MATERIALS AND METHODS

Plasmids. Plasmid pHUD 10-3, with the tetracycline operator and pUHD15-1, with the tetracycline activator were kind gifts from H. Bujard and K. Morishita. The *Eco*RI-*Bam*HI fragment of pUHD15-1 was inserted into pUC18 to yield pUC18tTA. The *Eco*RI-*Hind*III fragment, containing the tTA coding gene was excised from plasmid pUC18tTA and cloned into a plasmid pBK-CMV(pBK-CMVtTA). The rat *Tsc2* full-length cDNA sequence [without residues encoded by exon 25, 31 and CAG alternative splicing acceptor triplet of exon 26 (4)] and SV40 poly A signal fragment were cloned into

[1] To whom correspondence should be addressed. FAX: 81-3-5394-3815.

0006-291X/96 $18.00

pBluescript IISK+(pBSr*Tsc2* full+polyA), then the human cytomegalovirus minimal promoter sequence with the tetracycline operators derived from pUHD10-3 (PhCMV-1) was inserted upstream of pBSr*Tsc2* full+polyA (pTetOPr*Tsc2* full+polyA). pTetOPCAT contains the PhCMV-1 sequence upstream of the CAT reporter gene pCAT-Basic (Promega) (Fig. 1 A).

Immunoprecipitation. 5×10^5 of COS-7 were transiently transfected with 30 μg of supercoiled plasmid pSVFLAGr*Tsc2* or pSVFLAG control vector by the calcium phosphate precipitation method. After 48 hr incubation, cells were washed twice, maintained in culture medium for 72 hr and then labeled with [^{35}S] methionine. Cell lysates were subject to immunoprecipitation with anti-FLAG monoclonal antibody. Immune complexes were analyzed on 10% SDS/PAGE, and visualized by autoradiography.

RNA and DNA isolation, Northern and Southern analysis. DNA was extracted as described previously (10). DNAs (20 μg/lane) were digested with appropriate restriction enzymes, electrophoresed on a 0.8% agarose gel, and transferred onto nylon membranes (Biodine Transfer membrane; Pall BioSupport). These blots were hybridized overnight at 60°C in a solution containing 7% SDS, 1% BSA, 1mM EDTA, 0.2M NaHPO4 and ^{32}P-labeled DNA probes (10^6 cpm/ml) prepared by the random oligonucleotide-priming procedure. The filters were washed once in 2 × SSC for 15 min and twice in 0.2 × SSC at 65°C for 30 min, and visualized by autoradiography. Total cytoplasmic RNA was isolated from LK9D and LK9DtAr*Tsc2* cells by the guanidium thiocyanate method. Twenty micrograms of each RNA sample were analysed by northern blot hybridization as previously described (2). Following procedure were as well as described in Southern blotting.

Transfection. LK9D cells established from a primary kidney tumor of an Eker rat were cultured in medium as described (11). 20 μg of pTetOPr*Tsc2* were co-transfected with 2 μg of pBKCMVtTA into LK9D cells by the calcium phosphate coprecipitation method, and incubated for 48 hr in growth medium in the presence of 20 μg of tetracycline per ml, and then shifted to selection medium containing 800 μg of G418 per ml and 20 μg of tetracycline per ml. After 2 weeks, several colonies were seen in a 100-mm dish, and each of the G418-resistant colonies was expanded for further analyses.

CAT assay. LK9DtTAr*Tsc2* cells stably transfected with pBKCMVtTA and pTetOPr*Tsc2* were grown in the absence or the presence of various amounts of tetracycline (1, 5, 10 or 20 μg/ml). The reporter plasmids (8 μg of pTetOPCAT) were transfected into LK9DtTAr*Tsc2* by the calcium phosphate precipitation method. The CAT assay was performed essentially using the method of Gorman (12).

Morphological changes and growth curves. Aliquots of 5×10^4 LK9DtTAr*Tsc2* cells were seeded on 60 mm dishes in 4 ml of culture medium in the absence or the presence of tetracycline (2 μg/ml), and incubated for 4 days. Cells were photographed under phase contrast with a Diaphotomicroscope. Cells from triplicate dishes were counted every 3 or 4 days for two weeks with a hemocytometer. Results were calculated as the average of three dishes.

RESULTS

The expression of the cloned *Tsc2* cDNA in mammalian cells was examined by the immunoprecipitation method using anti-epitope tag antibodies. The expected size (about 200kDa) of *Tsc2* product was seen in cellular extracts from the COS-7 cells transiently transfected with the *Tsc2*-expressing plasmid (Fig. 1B).

To achieve stable *Tsc2*-transformants, ptetOPr*Tsc2* was co-transfected with pCMVtTA into LK9D cells. After transfection, several derivative clones resistant to neomycin were isolated and cultured in the presence of tetracycline. A representative clone, LK9DtTAr*Tsc2*-3 which decreased its growth rate after withdrawal of tetracycline for 4–5 days, was used for all subsequent experiments. In the Southern analysis, digestion with *Hind*III or *Eco*RI produced different digestion patterns from parental LK9D cells using either *Tsc2* specific or *tet O* specific probes, demonstrating that LK9D tTAr*Tsc2*-3 had integration of exogenous *Tsc2* cDNA (Fig. 2A).

In the CAT assay, a tetracycline concentration of 1 μg/ml was sufficient to achieve base-line repression (Fig. 2B). As shown in the northern analysis, in the presence of tetracycline, a *Tsc2* mRNA band was not observed in LK9DtTAr*Tsc2* cells. Strong induction of *Tsc2* mRNA was demonstrated after withdrawing tetracycline. In the parental LK9D cells, endogenously expressed gene product was not shown in either the absence or presence of tetracycline (Fig. 2C).

Although the morphology of the parental LK9D cells, grown as a monolayer, was essentially unchanged by withdrawing tetracycline, that of LK9DtTAr*Tsc2*-3 was markedly altered. In the presence of tetracycline (2 μg/ml), LK9DtTAr*Tsc2*-3 cells as well as LK9D cells, looked spindle-shaped, and sarcomatoid or spheroid-shaped. However, 4 days after removing tetracycline, the majority (70–80%) of the LK9DtTAr*Tsc2*-3 cells had a more uniform morphology (epithelioid cell) and became flattened and enlarged in diameter compared to the LK9D cells grown in the presence

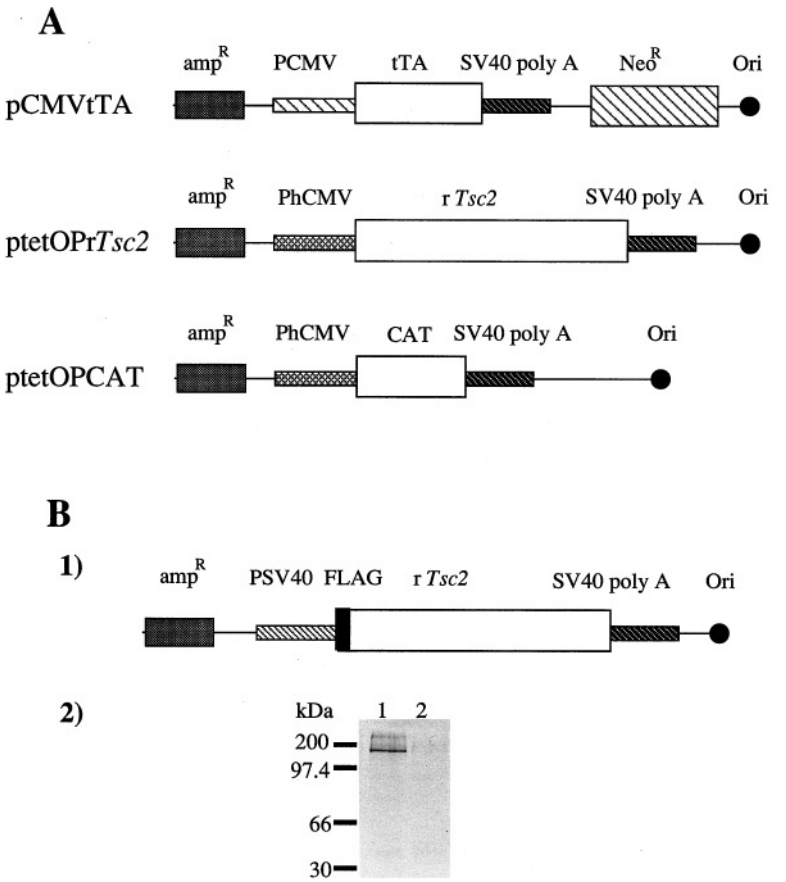

FIG. 1. *Tsc2* expression vectors. (A) The tetracycline-regulated system for expression of wild-type *Tsc2* and the CAT reporter gene. PhCMV; human cytomegalovirus promoter without the enhancer region fused to a heptad of tetracycline operators. pCMVtTA contains the tetracycline-regulated transactivator (tTA), driven by the entire cytomegalovirus promoter, including the enhancer region (PCMV). neo^R; neomycin resistance gene, pSV40; SV40 early promoter, SV40 poly A; SV40 polyadenylation signal, amp^R; ampicillin resistance gene, ori; origin of replication. (B) *Tsc2* cDNA expression in COS-7 cells. 1) Construction of the epitope-fused *Tsc2* cDNA expression vector. 2) Tsc2 protein identified by immunoprecipitation. Protein extracts were subjected to immunoprecipitation with anti-FLAG monoclonal antibody. lane 1; lysate from COS-7 transiently transfected with pSVFLAGr*Tsc2*, lane 2; lysate from COS-7 transiently transfected with pSVFLAG.

of tetracycline (Fig. 3A). The growth rates of the *Tsc2*-reconstituted LK9D cells were decreased to about a half in the absence of tetracycline as compared to the value in the presence of 5 μg/ml of tetracycline (Fig. 3B).

DISCUSSION

In the present study, in order to examine the significance of *Tsc2* inactivation in Eker rat renal carcinoma cells, we reconstituted a *Tsc2*-negative cell line. In the case of other tumor suppressor genes such as *p53*, which is known to be involved in programmed cell death, it has been reported to be difficult to establish stable transformants due to its cytotoxicity. Furthermore, constitutive expression of tumor suppressor genes might cause inhibition of proliferation during the reconstitution process. To avoid those possibilities, it is necessary to be able to regulate expression of transfected genes. For this purpose we used a well-controlled, tetracycline-operator system, which appears ideal for the conditional expression of genes whose products are cytostatic, and which has

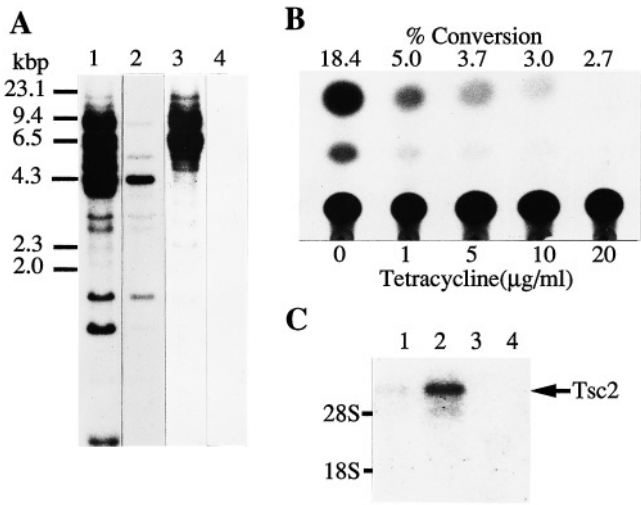

FIG. 2. Characterization of *Tsc2*-reconstituted LK9D cells. (A) Integration of the *Tsc2* cDNA gene. A Southern blot prepared using 10 μg of genomic DNA isolated from *Tsc2*-reconstituted LK9D cells (LK9DtTAr*Tsc2*) (lanes 1 and 3) or parental LK9D cells (lanes 2 and 4) and probed with rat *Tsc2* cDNA (lanes 1 and 2) or PhCMV specific sequence (lanes 3 and 4). (B) Dose response analysis of tetracycline action on LK9DtTAr*Tsc2* cells. LK9DtTAr*Tsc2* cells were transiently transfected with the CAT reporter plasmid, ptetOPCAT, and cultured in the presence of various concentrations of tetracycline as indicated. After 48 hrs after transfection, cells were harvested and assayed for CAT activity. (C) Regulated expression of *Tsc2* in LK9D cells. A northern blot prepared using 20 μg of total RNA isolated from LK9DtTAr*Tsc2* cells (lanes 1 and 2) or LK9D cells (lanes 3 and 4) cultured in the presence of 5 μg/ml of tetracycline (lanes 1 and 3) or in its absence (lanes 2 and 4). The blot was probed with rat *Tsc2* cDNA.

been applied to investigate functions of other tumor suppressor genes such as *p53* (13), *WT1* (14), and $p21^{waf1}$ (15).

Induction of the *Tsc2*-gene in *Tsc2*-negative Eker rat renal carcinoma cells altered their morphology dramatically (Fig. 3A). Thus, in the presence of tetracycline, *Tsc2*-reconstituted LK9D cells were spindle-shaped and resembled the parental cells. However, after withdrawing tetracycline from the culture medium, they became flattened and enlarged in diameter. This alteration in morphology presumably resulted from interaction between the *Tsc2* product and intracellular matrix molecules, leading to changes in the cytoskeleton.

We further demonstrated that overexpression of *Tsc2* results in a significantly reduced growth rate in the present growth-curve experiment (Fig. 3B). In the *Tsc2*-reconstituted LK9D cells, the expression of exogenously introduced-*Tsc2* gene in LK9D cells was very weak when examined by western blot and immunoprecipitation assays (data not shown). Although we have attempted to establish *Tsc2*-reconstituted clones by conventional gene expression system without an inducible promoter, no stable transformants expressing Tsc2 product at high levels could have been obtained (data not shown). These findings suggest that the Tsc2 protein, a putative tumor suppressor gene product, might have adverse effects on growth of recipient cells and only stable clones expressing the Tsc2 product at very low level could be expanded as dominant populations. The introduction of human chromosome 16, on which the *TSC2* gene is located, into a certain Eker rat renal carcinoma cell line supports this hypothesis (Dr. M. Oshimura; personal communication).

We conclude that instructive expression of *Tsc2* results in a decreased growth rate of LK9D cells. Such an ability to suppress the growth of tumor cells is a most important clue to understanding the biological function of the *Tsc2* gene. The mechanisms underlying *Tsc2*-mediated growth suppression are not yet elucidated and how this novel protein interacts, directly or indirectly, with the cell cycle clearly needs to be determined. The presently observed morphological changes suggest that *Tsc2* may be important for the organization of membrane-cytoskeletal elements. Recently we have

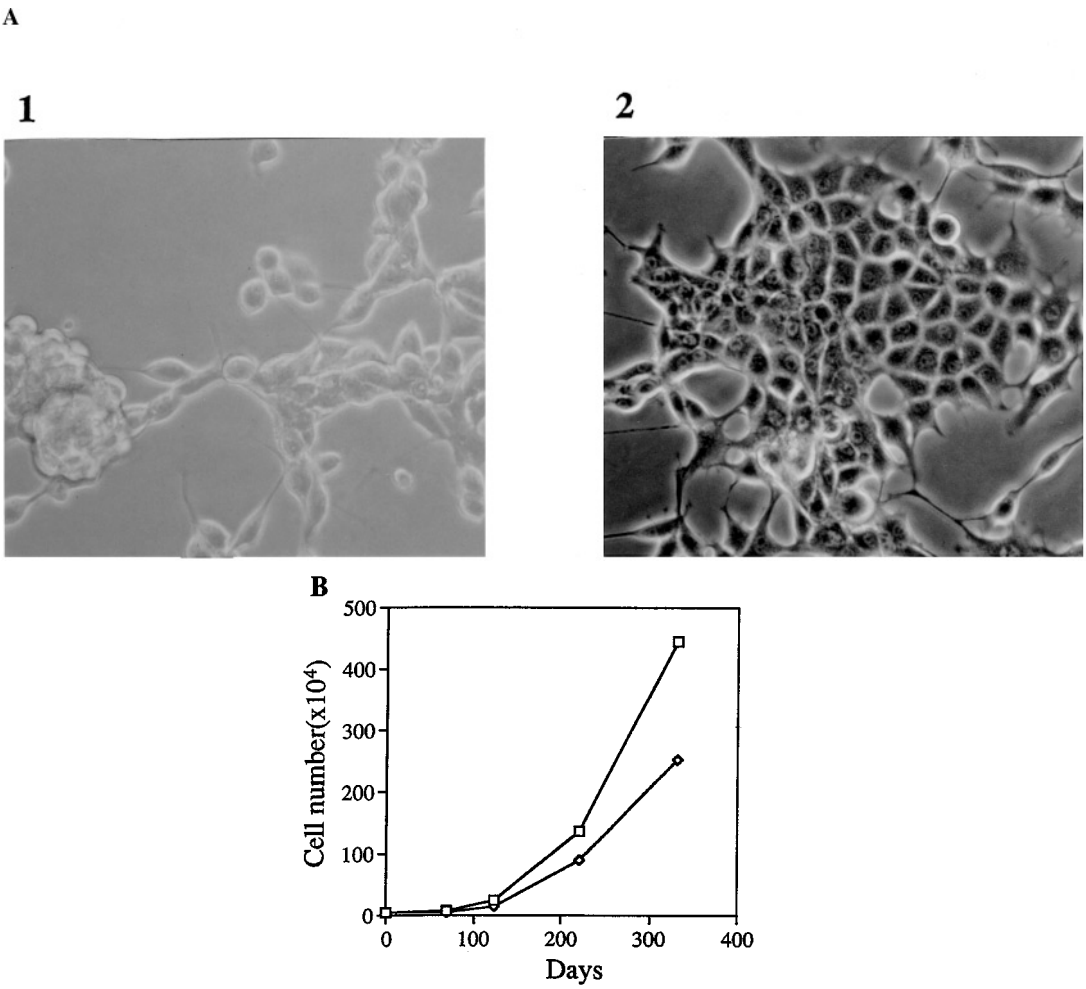

FIG. 3. Morphology and growth rate of cultured LK9DtTAr*Tsc2* cells. (A) Micrographs of representative LK9DtTAr*Tsc2* cells cultured for 72 hrs in the presence (panel 1) or absence (panel 2) of tetracycline. (B) Growth rate assay of *Tsc2*-reconstituted clones. LK9DtTAr*Tsc2* cells were seeded in 60mm dishes at 2×10^4 cells/plate in the presence (open squares) or absence (open diamonds) of tetracycline and live cells were counted in triplicate at 3- or 4-day intervals.

found that Tsc2 protein possesses transcriptional activation domains (16). We are now trying to identify suspected downstream effectors and associated molecules. The Eker rat continues to be a valuable experimental model for establishing the role of the *TSC2* gene in renal carcinogenesis.

ACKNOWLEDGMENTS

We gratefully acknowledge Drs. Alfred G. Knudson, Haruo Sugano and Tomoyuki Kitagawa for their encouragement throughout this work. The research was supported in part by Grants-in Aid for Cancer Research from the Ministry of Education, Science, and Culture of Japan and the Council for Tobacco Research of the U.S.A.

REFERENCES

1. Eker, R., and Mossige, J. A. (1961) *Nature* **189,** 858–859.
2. Hino, O., Kobayashi, T., Tsuchiya, H., Kikuchi, Y., Kobayashi, E., Mitani, H., and Hirayama, Y. (1994) *Biochem. Biophys. Res. Commun.* **203,** 1302–1308.
3. Kobayashi, T., Hirayama, Y., Kobayashi, E., Kubo, Y., and Hino, O. (1995) *Nature Genet.* **9,** 70–74.

4. Kobayashi, T., Nishizawa, M., Hirayama, Y., Kobayashi, E., and Hino, O. (1995) *Nucleic Acids Res.* (1995) **23,** 2608–2613.
5. Kubo, Y., Mitani, H., and Hino, O. (1994) *Cancer Res.* **54,** 2633–2635.
6. Kubo, Y., Klimek, F., Kikuchi, Y., Bannasch, P., and Hino, O. (1995) *Cancer Res.* **55,** 989–990.
7. The European Chromozome 16 Tuberous Sclerosis Consortium. (1993) *Cell* **75,** 1305–1315.
8. Wionecke, B., Konig, A., and Declue, J. E. (1995) *J. Biol. Chem.* **270,** 16409–16414.
9. Gossen, M., and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5547–5551.
10. Hino, O., Kitagawa, T., and Sugano, H. (1985) *Int. J. Cancer* **35,** 5–10.
11. Hino, O., Teata, J. R., Buetow, K. H., Taguchi, T., Zhou, J-Y., Bremer, M., Bruzel, A., Yeung, R., Levan, G., Levan, K. K., and Knudson, A. G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 327–331.
12. Gorman, C., (1985) *in* DNA Cloning (Glover, D. M., Ed.), pp. 143–165, IRL Press. Limitd, Oxford, UK.
13. Agarwal, M. L., Agrawal, A., Taylor, W. R., and Stark, G. R. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8493–8497.
14. Maheswaran, S., Englert, C., Bennett, P., Heinrich, G., and Haber, D. A. (1995) *Genes & Dev.* **9,** 2143–2156.
15. Chen, Y. Q., Cipriano, S. C., Arenkiel, J. M., and Miller, F. R. (1995) *Cancer Res.* **55,** 4536–4539.
16. Tsuchiya, H., Orimoto, K., Kobayashi, T., and Hino, O. *Cancer Res.* in press.