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# Differentially Expressed Genes in Activin-Induced Apoptotic LNCaP Cells

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**Gene transcripts differentially expressed in activin-induced human prostatic LNCaP apoptotic cells have been discovered by an improved subtractive hybridization method, uracil-DNA subtraction assay (USA), which involves digestion with uracil-DNA glycosylase and mung-bean nuclease. Among the five up-regulated and seven down-regulated genes, we have identified six known (>95% homology and similar size; p16, p53, Siva, RHAMM, Pax2, and eIF-4a1), three homologues (>95% homology but different size; myosin, a helicase motif, and a kinase motif), and three novel genes (no homology). In addition, anti-sense knock-out of a resulting novel kinase-like gene was found to abolish the apoptotic DNA fragmentation in activin-treated LNCaP cells. These findings indicate a new potential mechanism in DNA fragmentation of activin-induced cell-cycle arresting and apoptosis.** 

To this day, detection of genes differentially expressed in the same cell type under two different conditions has been achieved by methods such as differential display PCR (1) and representational difference analysis (RDA) (2, 3). However, these methods are technically difficult, time consuming and often result in a great number of identified homologues which are suspected to be differentially expressed. Therefore, we have explored a process to eliminate unwanted homologous sequences between compared cDNA libraries by using non-modified cDNAs as tester and uracil-incorporated cDNAs (U-DNAs) as driver. The elimination of non-differentially expressed homologues is achieved by hybridization of the tester to the compared driver and subsequent digestion of driver-bound hybrids with uracil-sensitive endonucleases. In this way, only differentially expressed genes in the tester can be preserved after the enzymatic digestion and final PCR amplification. To prevent cross-over digestion among gene transcripts with a small homologous domain, the compared cDNA libraries are preferentially restricted by a four-cutting enzyme (such as Hpa2) and ligated to a specific adaptor to yield sequences ranging from 100 to 700 base pairs with low complexity. This step also allows for a greater completeness during subtractive hybridization and differential amplification (2).

As a model for the USA detection of differentially expressed genes in apoptosis, we treated LNCaP cells, an androgen-sensitive human prostate cancer cell line, with activin. Previously, activin, a member of the transforming growth factor $\beta$ (TGF-$\beta$) superfamily, has been shown to inhibit cell proliferation and enhance apoptosis in these cells (4, 5). We have prepared an activin-treated LNCaP cDNA library as tester and an untreated one as driver, and vice versa, in which the driver always contained uridine. After subtractive hybridization of tester to driver and then enzymatic digestion, the differentially expressed genes were amplified and displayed on an electrophoresis gel, from which the results were extracted and confirmed by Northern blot analysis. Based on the sequence data of the results from a GenBank search, we have successfully identified twelve highly differentially expressed genes between the activin-treated and untreated LNCaP cells. However, since the identified novel genes may be involved in either cell-cycle arrest or apoptosis, the possible apoptosis-related genes were further tested by anti-sense knock-out transfection, in which knocking out a positive apoptotic gene will rescue the activin-treated cells from DNA fragmentation. All information so obtained shed light on the transcriptional control of activin-induced cell growth inhibition and apoptosis.

## MATERIALS AND METHODS

*Oligonucleotides.* Four oligonucleotides were used in the uracil-DNA subtraction assay as follows: Tester-24mer (5′-GCCACCAGAAGAGCGTGTACGTCC-3′), Tester-11mer (5′-CGGGACGTACA-3′ with a dephosphorylated 5′-end); Driver-24mer (5′-CGGTAGTGACTCGGTTAAGATCGC-3′), Driver-11mer (5′-CGGCGATCTTA-3′ with a dephosphorylated 5′-end). The 24mer oligonucleotides were used

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as 5′-adaptors and primers for differential PCR, while the 11mer oligonucleotides functioned as linkers for ligation of 5′-end adaptors.

*General methods.* All routine techniques and DNA manipulations, including gel electrophoresis, plasmid preparations and transformations, were performed according to standard procedures (6). All enzymes and buffer treatments were applied following the manufacture's recommendations (Boehringer Mannheim, Indianapolis, IN). For Northern blots, mRNAs were fractionated on 1% formaldehyde-agarose gels and transferred onto nylon membranes (Schleicher & Schuell, Keene, NH). Probes were labeled with the Prime-It II kit (Stratagene, La Jolla, CA) by random primer extension in the presence of [$^{32}$P]-dATP (>3000 Ci/mM, Amersham International, Arlington Heights, IL), and purified with Micro Bio-Spin chromatography columns (Bio-Rad, Hercules, CA). Hybridization was carried out in the mixture of 50% freshly deionized formamide (pH 7.0), 5 × Denhardt's solution, 0.5% SDS, 4 × SSPE and 250 μg/ml denatured salmon sperm DNAs (18 h, 42°C). Membranes were sequentially washed twice in 2 × SSC, 0.1% SDS (15 min, 25°C), and once each in 0.2 × SSC, 0.1% SDS (15 min, 25°C); and 0.2 × SSC, 0.1% SDS (30 min, 65°C) before autoradiography.

*Cell culture and activin treatment.* LNCaP cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum with 100 μg/ml gentamycin at 37°C under 10% $CO_2$. For three-day activin induction, LNCaP cells were treated with 200 ng/ml activin per day, while other cells were treated with medium as control. On the fifth day after the first treatment, a 56% reduction in growth was observed in the activin-treated cells compared to the control by both microscopy and cell counting as previously reported (4). The two groups of cells were independently trypsinized and mRNAs were purified by poly-(dT) dextran columns (Qiagen, Santa Clarita, CA). The quality of isolated mRNAs was assessed on 1% formaldehyde-agarose gels.

*Generation of double-stranded cDNA libraries and representative amplicons.* The first strand of cDNAs was prepared by reverse transcription of the mRNAs with oligo(dT) primers following the protocol of a cDNA Cycle kit (Invitrogen, Carlsbad, CA), and its quality was assessed on a 2% agarose gel. The second strand of cDNAs was synthesized with an enzyme cocktail containing DNA polymerase I, RNase H and $T_4$ ligase, as reported by Gubler et al. (7). To generate adequate lengths of cDNA amplicons for efficient subtraction and amplification, double-stranded cDNAs (1.5 μg) were digested with Hpa2 (3 hr, 37°C), recovered by 100 bp-cutoff microconcentrator columns (Amicon, Beverly, MA) and then ligated to either Tester-24/11mer or Driver-24/11mer adaptors in a mixture containing the 24/11mer oligo (0.75 nmol each) in 30 μl of 1 × ligase buffer. For precise ligation between restricted cDNAs and the adaptors, the mixture was held by gradually cooling from 50°C to 10°C over a period of one hour, and then $T_4$ ligase was added to anneal the 24mer oligonucleotides onto the 5′-ends of the restricted cDNAs, at 16°C for 14 hr. This formed the representative amplicons for both the tester and driver respectively, depending on the distinctive adaptor used.

*Generation of uracil-cDNA driver.* For incorporation of deoxyuridylate into the driver cDNAs, multiple PCR reactions were set up as follows: each 50 μl reaction containing driver amplicon (10 ng), 1 μM Driver-24mer oligo, dNTP mixture 1 (0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dCTP, 0.05 mM dTTP and 0.5 mM dUTP) and Taq DNA polymerase (5 U) in 1 × PCR buffer (Fig. 1). The Driver-11mer linker was melted away (3 min, 72°C), and the recessed 3′-ends were filled in with Taq DNA polymerase (3 min, 72°C). Thirty cycles of amplification were performed (1 min, 95°C; 3 min, 72°C), and the PCR products were combined and recovered by a microconcentrator column in Tris buffer (10mM, pH 7.0). The driver-adaptor was removed by Hpa2 cleavage and the digest was recovered by a microconcentrator column in EE × 3 buffer (30 mM EPPS, pH 8.0 at 20°C; 3 mM EDTA) at 1 μg/μl to form the driver. The quality of the driver (2 μg) was assessed on a 2% agarose gel, ranging from 100 bp to about 1 kb.

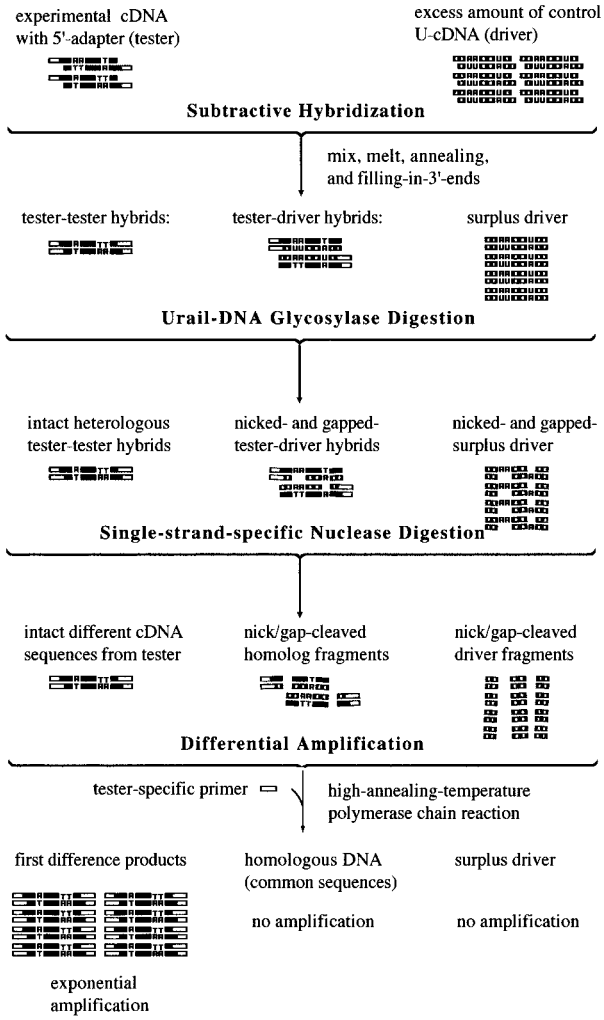



**FIG. 1.** Schematic protocol for uracil-DNA subtraction assay (USA), illustrating the sequential enzymatic digestion and differential amplification steps after subtractive hybridization. The enzymatic digestion contains two substeps: uracil-DNA glycosylase digestion which nicks all driver-like homologues, and single-strand-specific nuclease digestion which cleaves the homologues into unamplifiable fragments. The process is outlined through the final products of the first round of USA. To reiterate another round of subtraction, the first difference products are used as tester following the same scheme to generate the second difference products and so on.

*Subtractive hybridization, enzymatic digestion and differential amplification.* For the first subtractive hybridization, 0.2 μg of tester amplicon (tester) was mixed with 10 μl restricted driver, overlaid with mineral oil, denatured (5 min, 98°C) and immediately cooled on ice. Hybridization was performed with the phenol emulsion reassociation technique in a 400 μl solution containing 1.5 M NaSCN and 8% phenol at 25°C for 48 hr (8, 9). An emulsion of the phenol and aqueous phases was maintained throughout the hybridization by continuous agitation on a vortex mixer. The hybridized DNAs were recovered by a microconcentrator column in 20 μl of Tris buffer and treated with DNA polymerase I-$T_4$ DNA polymerase 3:1 mixture (3 min, 37°C without dNTPs; 25 min, 37°C with dNTP mixture 2 in 0.2 mM each for dATP, dGTP, dCTP and dTTP) to fill in the 3′-end of the tester.

In order to eliminate driver homologues in the hybridized DNAs, uracil-DNA glycosylase (UNG) was added (30 min, 25°C) to remove

uracil from driver-driver and driver-tester hybrid duplexes, resulting in a partially single-stranded conformation with abasic nicks and gaps. The abasic homologue duplexes were then subjected to digestion by a single-strand-specific endonuclease, such as mung-bean nuclease (MBN) and S1 nuclease (2 U at 25°C for 20 min), and finally cleaved into unamplifiable fragments (<50 bp). To ensure the completeness of homologue elimination, the hybridization and enzymatic digestion described above were repeated at least once. The final digest was recovered by a microconcentrator column in 20 $\mu$l of Tris buffer and prepared for differential PCR in a 50 $\mu$l reaction, containing 2 $\mu$l of the digest, 1 $\mu$M Tester-24mer oligo, dNTP mixture 2 and Taq DNA polymerase. Final PCR products were phenol-extracted, ethanol-precipitated, resuspended in 20 $\mu$l of Tris buffer and assessed on a 3% agarose gel. The DNA bands shown on the electrophoresis gel were excised, recovered by a gel extraction kit (Qiagen) and further purified by a 4% non-denaturing polyacrylamide gel. The processes of hybridization, enzymatic digestion and amplification were repeated until a clear banding pattern was observed.

*Cloning and sequencing of difference products.* Final products of USA were ligated to the pCR2.1 plasmid and transformed into INV$\alpha$F′ cells using a TA cloning kit (Invitrogen). Double-stranded plasmid DNAs were purified by miniprep spin columns (Qiagen), and sequenced by a Sequenase v.2 DNA sequencing kit (Amersham) with dideoxy-mediated chain termination. Resulting sequences were searched and compared to the Genbank database using the BLAST program from the National Institutes of Health (NIH).

*Anti-sense knock-out assay.* Following the single-stranded amplification method reported by Medori et al. (10), 100 ng of each USA product was used in a 100 $\mu$l PCR reaction containing 30 pmol of anti-sense primer, 0.3 pmol of sense primer, dNTP mixture 2, Taq DNA polymerase and 1.5 mM $MgCl_2$. A thirty cycle PCR amplification was carried through denaturation at 95°C, annealing at 55°C and extension at 72°C for 1 min in each step. The anti-sense PCR product was recovered by a microconcentrator column in Hepes buffer (20 mM, pH 7.4), and modified with a covalent modification reagent (Epiclone, Alhambra, CA) to introduce covalent bonding capability between the modified probe and its target nucleotide sequence. Transfection was carried out with the DOTAP liposomal transfection reagent (Boehringer Mannheim) using cationic liposome-mediated intracellular transport of the anti-sense probe (0.7 $\mu$g/ml) into activin-treated LNCaP cells. After three-days of activin treatment and two-days of incubation as described above, genomic DNAs were isolated by an apoptotic DNA ladder kit (Boehringer Mannheim) and assessed on a 2% agarose gel. The cell growth and morphology were also examined.

## RESULTS AND DISCUSSION

### *Identification of Differentially Expressed Transcripts between Activin-Treated and Non-Treated LNCaP Cells by USA*

As shown in Fig. 2, when an activin-treated cDNA library was used as the tester and an untreated cDNA library was used as the driver, three bands which contained thirteen gene fragments (Fig. 2, lane i) were obtained only in the activin-treated cells, including five transcriptional products whose levels were increased at least two fold above the controls. These products are a CCPK (cell-cycle check-point protein kinase)-like homologue (LT6), p16 (LT7), p53 (LT11), a Siva-like homologue (LT14) and a novel gene. On the other hand, when an untreated cDNA library was used as the tester and an activin-treated cDNA library as the driver, one thick band and two weaker bands which contain fifteen gene fragments (Fig. 2, lane h) were obtained only in the untreated cells, including seven transcriptional products which were decreased to as low as 45% of the controls. These products are a myosin-like homologue (LC2), hyaluronan receptor (LC3), a helicase motif-like homologue (LC9), Pax2 (LC12), eIF-4A1 (LC13) and two novel genes. The five up-regulated gene products represented the genes involved in enhancing activin-induced cell-cycle arrest and apoptosis (11, 12, 13, 14), whereas the seven down-regulated genes indicated that the expression of these genes would be reduced by cell cycle arrest or apoptosis (15, 16, 17, 18, 19).

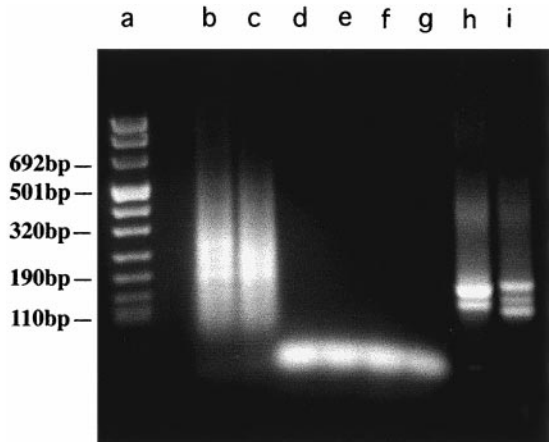
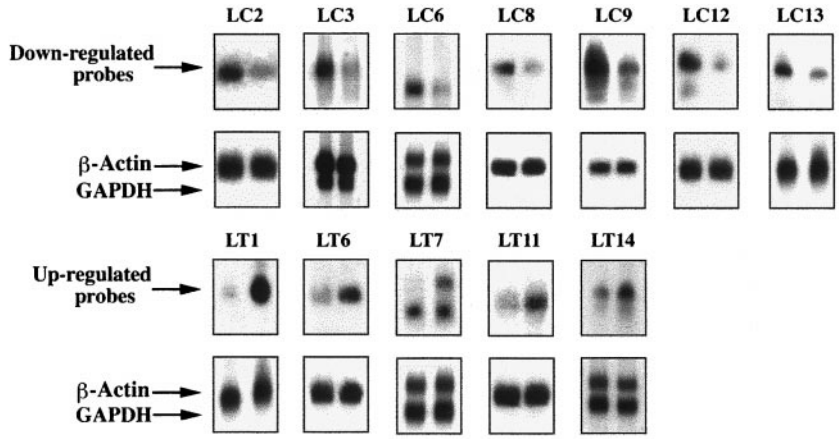



**FIG. 2.** Agarose gel electrophoresis of PCR subtracted products. From left to right: lane a, DNA markers; lane b, driver amplicon from untreated LNCaP cells amplified by driver-24 primer; lane c, tester amplicon from activin-treated cells amplified by tester-24 primer; lane d, driver amplicon amplified by tester primer; lane e, tester amplicon amplified by driver primer; lane f, driver amplicon subtracted with driver and then amplified by driver primer; lane g, tester amplicon subtracted with tester and then amplified by tester primer; lane h, driver amplicon subtracted with tester and then amplified by driver primer; lane i, tester amplicon subtracted with driver and then amplified by tester primer. The self-subtraction of driver to driver (f) and tester to tester (g) shows complete elimination of all sequences, while the mutual subtraction between tester and driver (h, i) presents difference products on the gel, indicating differential gene expressed in the tester and driver respectively. The misuse of PCR primer (d, e) will result in no amplification due to the specific affinity of the primer to its own adaptor.

Previously, p53 and p16 have been shown by us (13) and others (12) to be up-regulated in activin-mediated inhibition of cell proliferation and enhancement of apoptosis in LNCaP cells. Indeed, both genes were identified by the USA method and are known to be involved in the G1 phase arrest of the cell cycle as well as the induction of apoptosis. No sequence was found when a cDNA library was subtracted with itself (treated to treated and untreated to untreated, respectively) after thirty cycles of PCR amplification, suggesting that the identification of differentially expressed genes is highly specific. In addition, the cross-reaction be-




| USA Probes: | Gene (Size): | Homology %: | Change % (σ): | Function: |
|---|---|---|---|---|
| Down-regulated: | | | | |
| probe 1 (LC2) | myosin-like (1.1kb) | 99% | -47.6 (1.46)* | cytoskeleton |
| probe 2 (LC3) | RHAMM (2.8kb) | 95% | -55.9 (1.54)* | cytoskeleton |
| probe 3 (LC6) | novel (1.0kb) | | -60.9 (3.05)* | ? |
| probe 4 (LC8) | novel (2.0kb) | | -64.9 (3.09)** | ? |
| probe 5 (LC9) | helicase motif-like (1.3kb) | 95% | -60.5 (4.66)* | replication |
| probe 6 (LC12) | Pax2 (3.7kb) | 97% | -77.0 (2.37)** | proliferation |
| probe 7 (LC13) | eIF-4A1 (1.7kb) | 100% | -53.5 (0.00)* | translation |
| Up-regulated: | | | | |
| probe 8 (LT1) | novel (0.8kb) | | +728 (1.53)** | ? |
| probe 9 (LT6) | CCPK-like (1.8kb) | 100% | +265 (4.38)** | spindle lesion apoptosis |
| probe 10 (LT7) | p16 (0.7kb) | 97% | +354 (0.67)* | G1 arrest |
| probe 11 (LT11) | p53 (1.7kb) | 100% | +213 (5.35)** | G1 arrest |
| probe 12 (LT14) | Siva (1.0kb) | 100% | +191 (3.19)* | apoptosis |

* n=3, p<0.01
** n=4, p<0.01

**FIG. 3.** Autoradiogram of positive Northern blots hybridized to the final difference products of USA. Upper panel (LC2 to LC13) indicates seven down-regulated genes mainly present in untreated LNCaP cells but not in the activin-treated cells, while the lower panel (LT1 to LT14) shows five up-regulated genes significantly increased after activin treatment. p16 (LT7) and p53 (LT11) have been known to be up-regulated in the activin-treated LNCaP cells. The down-regulated known genes (LC2, 3, 9, 12, 13) are related to physiological functions of cells, and the up-regulated known genes (LT6, 7, 11, 14) are involved in either cell-cycle regulation, apoptosis or both. Genes listed are transcriptionally altered above two folds. The size of each identified gene transcript is deduced from individual Northern blots, and the homology shown here indicates the sequence similarity between the identified fragment and its deduced gene, rather than the full identified sequence.

tween tester and driver was completely inhibited by specially designed adapters and primers which were absolutely inert to each other under our PCR conditions (Fig. 2, lanes d & e). Moreover, there was no significant difference between the first round and the second round of USA in the present model, indicating that a complete subtraction was achieved. Nevertheless, when cDNA libraries derived from tissues, rather than cells, were used, more than one round of subtraction was required to obtain clear-cut results (data not shown).

CCPK, a serine-threonine kinase involved in regulation of the cell cycle, plays a role in genetic interaction between the regulation of mitosis, cell differentiation, and apoptosis (11, 21, 22). Siva is a proapoptotic protein and overexpression of this molecule resulted in apoptosis in various cell lines (14). A myosin regulatory light chain gene has been isolated from a normal human prostate library (23) which has been reported to block the TNF activation of DNA fragmentation (24). The receptor for hyaluronan-mediated motility (RHAMM) has been reported to mediate migration, transformation, and meta-

static spread of murine fibroblasts (16) and is expressed in breast cancer cells (25). Helicase motif is a gene that functions in the maintenance of genome stability and in the suppression of illegitimate recombination. The expression of Pax2 gene has been reported to reduce cell growth of renal carcinoma cells (18, 19). Eukaryotic translation initiation factor 4A1 is thought to be involved in tumor development (19, 26, 27).

As observed previously, the G1 phase arrest of the cell cycle occurs in the third day after activin treatment and apoptosis begins early in the fifth day. Presumably, the identified genes in our model are related to the G1 phase arrest and also the late activation phase of apoptosis. Our results demonstrate that most of the identified up-regulated genes are likely to be involved in either G1 arrest, apoptosis or both. We also have identified several novel genes which may also be involved in cell cycle arrest. Indeed, the identification of these genes is consistent with our previous observations that activin affects cell growth in LNCaP cells through the inhibition of cell proliferation and enhancement of apoptosis.

### *Confirmation of Differential Expression Levels by Northern Blot Hybridization*

As shown in Fig. 3, all identified genes were confirmed by Northern blot hybridization (N = 3 or 4) onto mRNA libraries from activin-treated and untreated cells, providing direct evidence for the alterations of gene expression between activin-treated and untreated LNCaP cells. A fragment showing at least two-fold changes ($P < 0.01$) was considered positive. Thirteen clones were identified from activin-treated cells and fifteen clones from non-treated control cells. Of which twelve positive clones showed 2-8 fold alterations of mRNA expression, among which five were up-regulated and seven down-regulated in activin-induced cells. As listed in Fig. 3, the five up-regulated genes are related to either cell-cycle arrest or apoptosis, and the seven down-regulated genes are involved in the maintenance of normal cell physiology. The mRNA length of a positive clone was deduced from the Northern blots and then used to match its original gene with sequence data from Genbank.

### *Functional Assay of Up-Regulated Novel Genes from Apoptotic Cells by Anti-Sense Knock-Out Transfections*

Since the difference products identified by USA are partial fragments restricted from the original gene transcripts, by using anti-sense knock-out transfection we have tested the functions of the difference products in preventing the apoptotic effects on activin-treated LNCaP cells. From USA results, clone LT6 (a CCPK-like homologue) was expressed only in apoptotic LNCaP cells while clone LC9 (a helicase-motif-like homologue) was expressed only in the proliferative LNCaP cells. As shown in Fig. 4, the anti-sense of LT6 rescued activin-treated LNCaP cells from apoptotic DNA fragmentation, whereas that of LC9 further increased the fragmentation. The sense control of both genes showed no influence on apoptotic DNA fragmentation, indicating that the effects of knock-out are sequence-specific. For operational purposes, clone LT6 was referred to as apoptosin whereas LC9 apoptostatin.

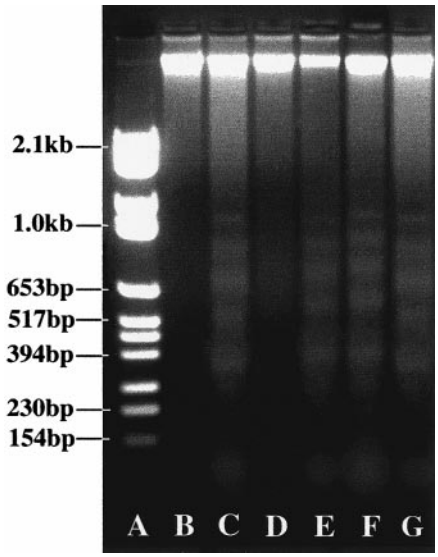



**FIG. 4.** Distinctive functions detected after anti-sense knock-out of LT6 and LC9. The antisense of LT6 rescues activin-treated LNCaP cells from apoptotic DNA fragmentation (lane D), whereas that of LC9 increases the fragmentation (lane F). Both transfection of control and sense sequences shows no effect on apoptotic DNA fragmentation (lane C, activin-treated positive control; lane E, result of sense-LT6 transfection; lane G, result of sense-LC9 transfection). The result of antisense-LT6 transfection is similar to normal genome from untreated cells (lane B), indicating a significant inhibition of apoptotic DNA fragmentation after blocking the gene transcript of LT6 in activin-treated LNCaP cells.

It is noteworthy that the results presented demonstrate a totally new approach in our understanding of apoptosis as wells as genes in cancer development and treatment. The full-length mRNA sequence of apoptosin and apoptostatin as well as the functions of these genes in normal and cancer cells remain to be defined. Nevertheless, the USA method provides a more efficient and sensitive means for identifying differentially expressed genes. Potentially, this method can be used in identifying different gene expressions involved in development, cell differentiation, aging, and a variety of pathological disorders, such as cancer formation, genetic defects, autoimmune diseases, and any other disorders related to genetic malfunction. The identification of these differentially expressed genes will help in the determination of their open-reading frames and corresponding peptides which may contribute to a specific drug-design or therapy for regulation of the expression of these genes.

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