RETINOIC ACID INDUCES THREE NEWLY CLONED HOXA1 TRANSCRIPTS IN MCF7 BREAST CANCER CELLS

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SUMMARY: Coordinated expression of genes involved in development, differentiation and malignant transformation is regulated by transcription factors including homeodomain-containing proteins. However, most of their cDNA sequences are still unknown. We report here the molecular characterization of three newly cloned HOXA1 transcripts from human breast cancer cells. In addition, we provide evidence that these alternatively spliced transcripts encode one homeodomain-containing protein and two products lacking the conserved DNA-binding domain. Moreover, we demonstrate that all three HOXA1 transcripts are induced by retinoic acid in MCF7 cells. Taken together, our results suggest that HOXA1 gene may be a key element in the establishment of the breast cancer cell phenotype. © 1995 Academic Press, Inc.

Homeodomain-containing proteins control, as transcription factors, the expression of multiple genes implicated in development and differentiation (1). Homeobox genes share a highly conserved 183bp region that codes for a 61 amino acid DNA binding domain called "homeodomain" (2). These genes were initially discovered in Drosophila where they control segment identity (3). Homologous homeobox genes have since been cloned in multiple species, including human (4, 5). To date, 38 human class I homeobox genes have been identified. They are organized in four clusters (HOX loci A, B, C and D) that are located on chromosomes 7, 17, 12 and 2 respectively (6) and are coordinately expressed in a spatiotemporal fashion (7). Because of its chromosomal localization, the HOXA1 gene may play a key role in the modulation of expression of other HOX genes. Indeed, in the developing embryo, murine homologue Hox-a1 protein may regulate the expression of other Hox genes in its rostral domain of expression, as suggested by targeted deletion experiments

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Abbreviations used:
RT-PCR, reverse transcriptase polymerase-chain reaction; Class I, homeoproteins sharing an Antennapedia-like homeodomain; DMEM, Dulbecco's modified Eagle medium; RA, retinoic acid.

(8). Beside this function in development, recent studies have demonstrated expression of HOXA1 gene in a variety of adult tissues (9-13). Moreover, in the murine system, ectopic expression of Hox-a1 in transfected rodent cells induced tumorigenesis (14) whereas upregulation of the murine Hox-a1 gene has been observed in neoplastic mammary glands (15). These studies suggest that this homeodomain-containing protein may play a role in cellular transformation and/or in tumor progression.

The aim of this study was to perform molecular characterization of HOXA1 mRNAs and to investigate the expression of this HOX gene in human breast cancer cells. We report here the cloning of three new HOXA1 transcripts. Moreover, we provide evidence that these transcripts are generated by alternate splicing events and presumably encode one homeodomain-containing protein and two truncated products lacking the conserved DNA-binding domain. We further demonstrate that all three HOXA1 transcripts are modulated by retinoic acid in MCF7 breast cancer cells. Taken together, our results suggest that HOXA1 may play a central role in the establishment of the breast cancer cell phenotype.

MATERIALS AND METHODS

Cell culture and treatments
MCF7 cell line, obtained from the American Type Tissue Collection (Rockville, MD) were maintained in "DMEM" containing Dulbecco's modified Eagle medium with 10% fetal calf serum (Gibco, Grand Island, NY) and antibiotics (100 µg/ml). For retinoic acid treatment, trans RA (Amersham, Airlington Heights, IL) was added to the cultures as concentrated stock solutions in pure ethanol (0.1% of the total volume) to reach a final 10^{-6}M concentration. Untreated cells were exposed to the same amount of ethanol.

Screening of the MCF7 cDNA library
An MCF7 cDNA library in UniZap XR phage DNA (Stratagene, La Jolla, CA) was constructed and screened with a nick translated [^{32}P] labeled 102 bp cDNA fragment encoding a partial homeodomain region of HOXA1 obtained as described (16). Positive clones were sequenced in both strands by the dideoxy method using the Sequenase Version 2.0 kit (United States Biochemicals, Cleveland, OH).

HOXA1 and actin cDNA synthesis
Total cellular RNA was isolated by the guanidium isothiocyanate extraction procedure and cesium chloride gradient centrifugation (17). cDNA synthesis was performed at 42°C for 30 minutes using 1µg or 0.1µg total cellular RNA (for HOXA1 and actin cDNA synthesis respectively) using Superscript reverse transcriptase (Gibco BRL, Gaithesburg, MD) and a 3' specific primer (5'-GTTGTCTGGGGCTGGAGCC-3' for HOXA1 cDNA synthesis or 5'-CTGCATCGTGTCGAAGAG-3' for actin cDNA synthesis (18)). The RNA template was degraded by RNase H (Gibco BRL). The cDNAs were then purified through GlassMAX Spin Cartridge (Gibco BRL). All reactions were performed in parallel in the absence of reverse transcriptase to verify dependence on an RNA template for subsequent PCRs.

Cloning of HOXA1 5'end cDNAs
HOXA1 5'end cDNAs were PCR amplified using a HOXA1 specific downstream primer (5'-CGTACTCTCCAACTTTCCC-3') and a primer located upstream of the initial ATG of the murine Hox-a1 cDNA (5'-ACTCAGTGACAGATGGAC-3') (19). PCR steps included an initial denaturation step at 95°C for 3 minutes ("Hot Start"), 45 seconds at 80°C to allow the addition of the Taq polymerase, 35 cycles of denaturation (95°C for 60 sec), annealing (55°C for 60 sec) and extension (72°C for 2 min) followed by a final extension at 72°C for 5 min. The resulting amplified fragments were purified through Midi-SELECT Spin columns and ligated into pNoTA vector using the "prime PCR Cloner Cloning System" kit (5 Prime-3

Prime, Boulder, CO), according to the protocol provided by the manufacturer. The resulting clones were sequenced as described above.

Semi-quantitative PCRs

HOXA1 and actin cDNAs (see above) were amplified using either HOXA1 primers (5'-ATGAACTCCTTCCTGGAATA-3' and 5'-CGTACTCTCCAACTTTCCC-3') or actin primers (5'-ATGATATCGCCGCGCTCG-3' and 5'-CGCTCGGTGAGGATCTTCA-3' (18)) located on different exons in both cases. HOXA1 and actin PCR amplifications included 35 and 25 cycles, respectively and were identical to those described above. The PCR products were submitted to Southern blotting using [^{32}P] labeled HOXA1 or actin cDNA fragments as probes. For semi-quantitative analysis, titration curves for both HOXA1 and actin cDNAs were prepared to determine the linear range of both amplifications (20). To avoid the plateau phase, the semi-quantitative RT-PCR experiments were performed with 5 μl of HOXA1 and actin cDNA solutions as templates (data not shown). The intensities of hybridization signals were quantified by the Scan Analysis program for the Macintosh (Biosoft, Cambridge, UK).

RESULTS AND DISCUSSION

Identification of a HOXA1 cDNA clone from MCF7 cells

We have previously amplified multiple partial homeodomains from MCF7 RNA by reverse transcriptase polymerase-chain reaction ("RT-PCR"), using primers within the highly conserved region (16). One of these was identified as representing the HOXA1 sequence. To obtain the full-length human clone, a phage MCF7 cDNA library was constructed and screened using the HOXA1 partial homeodomain sequence as a probe. Positive clone H304 contained 1619 bp of sequence (plus a poly(A) tail) which showed high homology with murine Hox-a1. According to current homeobox gene nomenclature (21), our sequence corresponds to HOXA1, the human homologue of murine Hox-a1 cDNA. By comparison with this murine homologue, clone H304 contained an open reading frame of 164 codons, including the homeopeptide, a conserved domain found in many homeodomain-containing proteins (bases 604-618 of Figure 1), but lacked the amino-terminal 166 codons.

Alternate splicing leads to three HOXA1 transcripts

Cloning of 5' HOXA1 sequences were performed by RT-PCR using a 5' primer from a murine Hox1.6 cDNA sequence located upstream of the initial ATG codon. As illustrated in Figure 2A, three fragments were amplified, designated A, B and C (450, 650 and 1150 bp, respectively, as estimated by comparison with a DNA marker on an agarose gel) and cloned for sequence analysis. No fragments were generated when reverse transcriptase was omitted from the cDNA synthesis reaction, demonstrating that the amplified fragments were originated from mRNA sequences and not contaminating genomic DNA (data not shown). Figure 1 represents a composite of the combined overlapping sequences of the three fragments together with the original clone H304. The sequence of the 5' end of fragments A, B and C was identical and included an initiating ATG codon (bases 10-13, see Figure 1) in a position analogous to the murine Hox-a1 cDNA. However, the internal sequences of the three 5' fragments did not completely overlap. By comparison with the murine Hox-a1 sequence, the major HOXA1 transcript would be represented by overlapping fragment B with the original sequence of clone H304, resulting in a 2159 base long mRNA
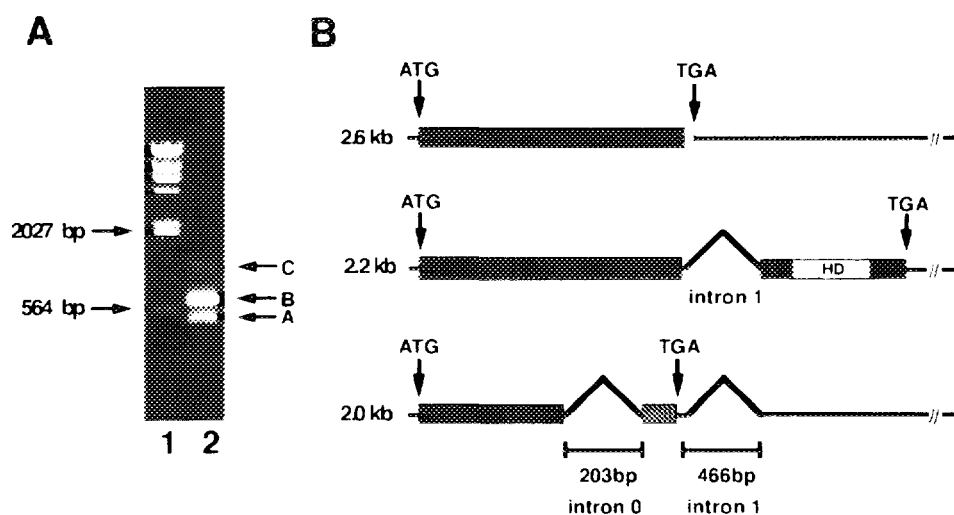
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                                                           AATGCAAGA        9
ATG AAC TCC TTC CTG GAA TAC CCC ATA CTT AGC AGT GGC GAC TCG GGG ACC TGC TCA GCC CGA GCC TAC CCC TCG   84
Met Asn Ser Phe Leu Glu Tyr Pro Ile Leu Ser Ser Gly Asp Ser Gly Thr Cys Ser Ala Arg Ala Tyr Pro Ser

GAC CAT AGG ATT ACA ACT TTC CAG TCG TGC GCG GTC AGC GCC AAC AGT TGC GGC GGC GAC GAC CGC TTC CTA GTG   159
Asp His Arg Ile Thr Thr Phe Gln Ser Cys Ala Val Ser Ala Asn Ser Cys Gly Gly Asp Asp Arg Phe Leu Val

GGC AGG GGG GTG CAG ATC GGT TCG CCC CAC CAC CAC CAC CAC CAC CAC CAT CAC CAC CCC CAG CCG GCT ACC TAC   234
Gly Arg Gly Val Gln Ile Gly Ser Pro His His His His His His His His His His Pro Gln Pro Ala Thr Tyr

CAG ACT TCC GGG AAC CTG GGG GTG TCC TAC TCC CAC TCA AGT TGT GGT CCA AGC TAT GGC TCA CAG AAC TTC AGT   309
Gln Thr Ser Gly Asn Leu Gly Val Ser Tyr Ser His Ser Ser Cys Gly Pro Ser Tyr Gly Ser Gln Asn Phe Ser

GCG CCT TAC AGC CCC TAC GCG TTA AAT CAG GAA GCA GAC |GTA AGT GGT GGG TAC CCC CAG TGC GCT CCC GCT GTT   384
Ala Pro Tyr Ser Pro Tyr Ala Leu Asn Gln Glu Ala Asp|Val Ser Gly Gly Tyr Pro Glu Cys Ala Pro Ala Val

TAC TCT GGA AAT CTC TCA TCT CCC ATG GTC CAG CAT CAC CAC CAC CAC CAG GGT TAT GCT GGG GGC GCG GTG GGC   459
Tyr Ser Gly Asn Leu Ser Ser Pro Met Val Gln His His His His His Gln Gly Tyr Ala Gly Gly Ala Val Gly

TCG CCT CAA TAC ATT CAC CAC TCA TAT GGA CAG GAG CAC CAG AGC CTG GCC CTG GCT ACG TAT AAT AAC TCC TTG   534
Ser Pro Gln Tyr Ile His His Ser Tyr Gly Gln Glu His Gln Ser Leu Ala Leu Ala Thr Tyr Asn Asn Ser Leu

TCC CCT CTC CAC GCC AGC CAC CAA GAA GCC TGT CGC TCC CCC GCA TCG GAG ACA TCT TCT CCA GCG CAG ACT TTT   609
Ser Pro Leu His Ala Ser His Gln Glu Ala Cys Arg Ser Pro Ala Ser Glu Thr Ser Ser Pro Ala Gln Thr Phe
                         Pro Pro Arg Ser Leu Ser Leu Pro Arg Ile Gly Asp Ile Phe Ser Ser Ala Asp Phe *

GAC TGG ATG AAA GTC AAA AGA AAC CCT CCC AAA ACA |GGT CAG TCC TGC TGG TTG GTG GAT GCT CCT TGA TTA TTC   684
Asp Trp Met Lys Val Lys Arg Asn Pro Pro Lys Thr |Gly Gln Ser Cys Trp Leu Val Asp Ala Pro **

TGG AAG GAG CTG GTA TGC TTA ATT TCC AAG GAA ATT AAT AAT GAT CTT TTT TTT AAA AGG CTT TTG TAT CAG ACT   759
AGT GTT GTA GTG CAT GGA GAG GGT GCC CAG AGG TGT TGG GGC AAA GAA GTC TCA GGG ATT GGT GTG TTT TCA AGG   834
ATT TTA TAC ATT TGG GAA ATA GGA AGT ATG TGG GGG TGG TTA TTG TGA CGG TAG TGT TCT GTT AAT ATC TTG AGG   909
CTC CAT TAA TCA CCG GTC TGA CCA TGC TAA TGG TTT ACA TCA GGT TAA CAC TTT ACA CCT CAT CAC TCA CCC TCC   984
ACA GCC CGA TTT GTG CTG AGT TCT TGA TCT TTT CTT TAC CTG AGT GTT GCC ATG AAG CGT GTG GGG AAT CTC AAG  1059
TCG ATT TAA AAA TTT TTA CAT TTC CGT CTC ATG GCT TCC CAC TTT GCC CCA CGG AAA GTT GGA GAG TAC GGC TAC  1135
                                                                       Gly Lys Val Gly Glu Tyr Gly Tyr

CTG GGT CAA CCC AAC GCG GTG CGC ACC AAC TTC ACT ACC AAG CAG CTC ACG GAA CTG GAG AAG GAG TTC CAC TTC  1210
Leu Gly Gln Pro Asn Ala Val Arg Thr Asn Phe Thr Thr Lys Gln Leu Thr Glu Leu Glu Lys Glu Phe His Phe

AAC AAG TAC CTG ACG CGC GCC CGC AGG GTG GAG ATC GCT GCA TCC CTG CAG CTC AAC GAG ACC CAA GTG AAG ATC  1287
Asn Lys Tyr Leu Thr Arg Ala Arg Arg Val Glu Ile Ala Ala Ser Leu Gln Leu Asn Glu Thr Gln Val Lys Ile

TGG TTC CAG AAC CGC CGA ATG AAG CAA AAG AAA CGT GAG AAG GAG GGT CTC TTG CCC ATC TCT CCG GCC ACC CCG  1362
Trp Phe Gln Asn Arg Arg Met Lys Gln Lys Lys Arg Glu Lys Glu Gly Leu Leu Pro Ile Ser Pro Ala Thr Pro

CCA GGA AAC GAC GAG AAG GCC GAG GAA TCC TCA GAG AAG TCC AGC TCT TCG CCC TGC GTT CCT TCC CCG GGG TCT  1437
Pro Gly Asn Asp Glu Lys Ala Glu Glu Ser Ser Glu Lys Ser Ser Ser Ser Pro Cys Val Pro Ser Pro Ala Ser

TCT ACC TCA GAC ACT CTG ACT ACC TCC CAC TGA GGCGGCTCCAGCCCCAGACAACAGCCCAGGCATCTCCTTGGGCTGGGACTTCTTA  1525
Ser Thr Ser Asp Thr Leu Thr Thr Ser His ***
CCCAAAGCACATGCTTAGCTTATCTTTCTTTCCATTTACAGTCTCTTTCTTCCTTTCTAATCCTATCTGGGGAGCTCCTGGCCAGGATAATATATTTGC  1624
AGATAATTCTGGACCAGAGACTTGGTGCGGGGTTAACACCTTCATCCAGATTGGGTGCCAGCATACATTTTCTGGTGGGCCTTAACATCCCTCCTGCTT  1723
TTAGGAGAATTCACAGAACCTACTGTTCCTTTCAGATGACCTTTTGGAAAATAGTTCCCTTTGCCAACAGAAACATGCCAGAAGGAATGTTCTCATCTT  1822
TTTTATCTAACTATATGTACAGCTCTCCCCTCCCTTGTCCTTGAAAGTAGGATATAGCGAAAGGCGAGTCCAGGAGCTCAGGAAGAAGAGATGCACTAT  1921
ATGTTTACACAATTAATTCATCCCTTAATTTAAGTCATTTTCATGTGTGTGAGTTTGCTGGTTGTAAATACTTTGTCCTAAGAGATTTATCTTTATACA  2020
GATTTTCTAGAAATGTTTAGGTTACTAAAACAGGGTGGGCAAACTCTCTAAACTGGTACAATTTTATAGGTGAAAGAAAAAATTCCCTCATTTAAACCC  2119
AATCAGATGCCTCAGAGGGTAGCCTTGATTTGTTCTTACAGTTAAGAAGCCCTGCAGAGCACAAACTTCAGAAACCCGGCTTCCTGTGCTAAGTCTTTC  2218
CCAATCTCTACCCCTTTCTTCTCGGGCCACCCTCTGTTTAAAATTTGTGCTGGGTTATTCAGAACCTAAAAGTATTATTCAAACCAATTTCTTCCTTCC  2317
ACAGTTATCTTAGCTGGATATAATGTATTTTCAGCTCAATTGTTAATGTGATGGATGGCACAATGAATGTATATTTTGTGTTATTCGTGAATAGTCTTT  2416
TGCATGTCGCACAATGTTTGATGTCCCAAAGTACCACACTGAGTTCTATCAGTTATCCTTTGTGAGCCTATGATATTCCCCATTTCCTGTACAATCAT  2515
GAACAGCTCTGAGATCCTGGAGTGATATGATCCAGAGCAGAGTTTACGGGTCTTAGGATGTCTGTAATAAATAAATATACTCAAAAAAAAAAAAAAAAA  2614
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Figure 1.

Complete nucleotide sequences of the three HOXA1 transcripts and their predicted amino acid sequences. The homeobox region is underlined (thick underline). The 203 bp long sequence that is deleted in the shortest transcript is illustrated within the first boxed region. The 466 bp sequence that is present in the longest transcript is represented within the second boxed region (thick line). The polyadenylation signal is underlined (thin underline). The numbers in the right margin represent bases initiating from the cloned 5' untranslated region of all HOXA1 mRNAs. cDNA clone H304 encompasses bases 507-2614 with bases 646-1110 spliced out. The amino acid sequence of the three predicted HOXA1 open reading frames are identical up to Asp-113. The amino acid sequence of the 14 kilodalton HOXA1 protein resumes with a frameshift after the first boxed region and ends at the TGA * at position 609. The amino acid sequence of the 24 kilodalton HOXA1 protein encoded by the longest transcript continues through the first boxed region and ends within the second boxed region at the TGA ** at position 676. The amino acid sequence of the full-length 36 kilodalton HOXA1 protein encoded by the 2159 bp transcript continues through the first boxed region, resumes after the second boxed region and ends with the TGA *** at position 1468. The conserved homeopeptide sequence is represented by bold characters (codons 199-203).

Figure 2.
A. Amplification of HOXA1 5' end cDNAs. Three amplified fragments were obtained (lane 2) and named fragments A, B and C (450, 650 and 1150 bp, respectively, as estimated by comparison with a DNA marker (lane 1)). B. Schematic representation of the three HOXA1 transcripts. Open reading frames are represented by rectangles whereas untranslated sequences are indicated by straight lines. Introns are illustrated with lines drawn at 45° angles. The homeodomain is represented by a white rectangle labeled "HD". The stipled rectangle in the 2.0 kb transcript represents a frame shift in the reading frame due to alternate splicing of intron 0.

with an open reading frame of 990 bases, and a predicted protein of 36 kilodaltons harboring both homeopeptide and homeodomain conserved sequences.

The shortest 5' fragment generated by PCR (fragment A) lacked 203 bp within the cDNA open reading frame (bases 349-551, first boxed region of Figure 1) and, most likely represents the 5' end of a differently spliced HOXA1 transcript. Although the sequences flanking the 203 bp are not consistent with a classic donor-acceptor splice site, the 5' and 3' ends of the 203 bp sequence (GT and AG, respectively) fit the consensus for the beginning and the end of an intron. The putative splicing event would cause a frameshift in the mRNA reading frame, leading to the production of a truncated 14 kilodalton protein lacking both the homeopeptide and the homeodomain due to the appearance of a termination codon TGA at position 609-611. Thus, a protein derived from the alternate splice would be identical to HOXA1 for the first 113 amino acids and then diverge for an additional 19 amino acids. A similar phenomenon has been described for the murine Hox1.6 gene (19).

In contrast to fragment B, fragment C contained an additional 466 bp sequence that was positioned analogously to the murine Hox-a1 intron (19). This region included multiple stop codons. Since amplification of this fragment from the mRNA template was reverse transcriptase-dependent (see above), it is unlikely that it was generated from contaminating genomic DNA. When combined with our MCF7 cDNA clone, this fragment represents an additional 2.6 kb HOXA1 transcript. A 24 kilodalton protein derived from this larger transcript would be identical to HOXA1 for the initial 212 amino acids, including the

homeopeptide sequence, and diverge for an additional 10 amino acids before termination due to a TGA stop codon. As with the protein that derives from the shortest transcript, it would not contain a homeodomain.

Figure 2B illustrates the alternate splicing event generating the three HOXA1 transcripts. The 203 bp sequence deleted in the 2.0 kb transcript is named "intron 0" (first boxed region of Figure 1) whereas the 466 bp sequence deleted in the 2.0 kb and 2.2 kb transcripts is named "intron 1" (second boxed region of Figure 1). According to this scenario, there are two potential introns in the HOXA1 gene. The 203 bp intron 0 does not contain any stop codons, and presumably is not spliced out in the 2.2 and 2.6 kb transcripts. The 466 bp intron 1 contains multiple stop codons, and must be spliced out to permit the translation of the homeodomain. The 2.2 kb transcript is thus the only HOXA1 mRNA that would generate the full-length HOXA1 protein. The physiological role of the potential truncated HOXA1 proteins remains unclear. It has been proposed that they could compete with the full-length protein for interaction with other transcription factors on their target genes (19). Recent reports have illustrated that cooperative protein-protein interactions may influence the transcriptional properties of HOX proteins (22, 23). They demonstrate that the biological specificity of each homeotic protein may be achieved, at least in part, through distinct interactions mediated by regions within or outside the conserved homeodomain. Interestingly, both potential truncated HOXA1 proteins lack the homeodomain whereas the homeopeptide that is present in the 24 kilodalton product is absent in the 14 kilodalton polypeptide. Since this conserved domain is involved in some protein-protein interactions (23), this phenomenon may reflect distinct transcriptional properties for both potential truncated HOXA1 products.

Induction of HOXA1 transcripts by RA in MCF7 cells

To investigate whether HOXA1 gene is modulated during retinoic acid-induced differentiation in breast cancer-derived cells, we performed semi-quantitative RT-PCR experiments using RNA from MCF7 cells treated with this drug. Confirming our sequencing results, three fragments of 1121 bp, 655 bp and 452 bp corresponding to the three transcripts described above, were observed on HOXA1 Southern blots (Figure 3A). All three HOXA1 transcripts were rapidly induced and displayed maximum expression after 72 hours of treatment. As expected, actin levels of expression remained constant in these cells (Figure 3B). HOXA1 gene has been shown to be rapidly induced by retinoic acid in the human teratocarcinoma cell line NT2/D1 as well (24). Taken together, these observation suggest a common mechanism for HOXA1 induction by retinoic acid independent of cell specific phenotypes. Our results demonstrate that three HOXA1 transcripts and presumably three HOXA1 proteins are affected by such treatment. Identification of target genes regulated by these polypeptides should lead to a better understanding of the cellular response to retinoic acid. We have detected HOXA1 expression in MCF7 cells as well as in multiple breast cancer tissues (data not shown). Our results, combined with Hox-a1 expression in mouse neoplastic mammary glands (15), suggest that HOXA1 may play a key role in the establishment of the breast cancer cell phenotype.
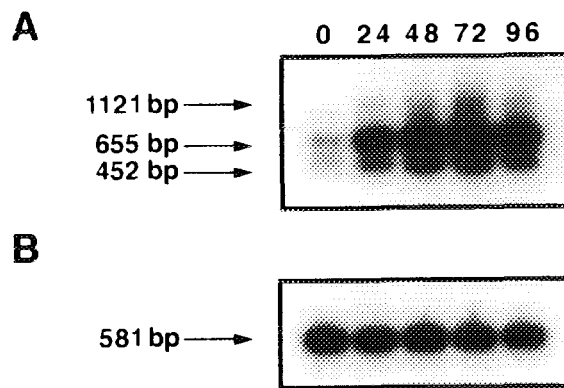
Figure 3.
HOXA1 and actin semi-quantitative RT-PCR experiments on MCF7 cells treated with RA.
A. Southern blot performed with a HOXA1 cDNA probe after RT-PCR of HOXA1 mRNAs.
B. Southern blot with β actin used as an internal control for the semi-quantitative RT-PCRs.
Size of HOXA1 and actin amplified fragments are illustrated. Hours of treatment are above
the autoradiograms.

ACKNOWLEDGMENTS

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