hydrogen bonding network may result in delocalization of the positive charge on the NH_2-terminal NH_3^+, and the removal of the hydroxyl group of either Tyr^7 or Tyr^{171} could therefore destabilize the complex by disrupting this network. Thus, an intact hydrogen bonding system may be essential to bind an NH_3^+ in this region. Additionally, the binding of NH_3^+ could signal a conformational change that would lock the peptide nonamer into the site, particularly since the tyrosines involved are derived from all major structural elements forming the cleft (Tyr^{59} on the α1 helix, Tyr^{171} on the α2 helix, and Tyr^7 on the floor of the cleft). In either case, the disruption of the hydrogen bonding system would result in a large change in binding affinity (measured indirectly in this case by the CTL titration value), as observed.

In the atomic model, the hydroxyls of Tyr^{84} and Thr^{143} are positioned to hydrogen bond to the terminal carboxylate group of a short peptide, and therefore presumably to the last carbonyl group in the binding site of a longer peptide (5). The failure of the mutations Y84F and T143V to affect CTL recognition (Fig. 3) is not an unexpected result of the removal of a single hydrogen bond and may suggest a role in the mutants for water molecules in replacing the hydrogen bonds to the negatively charged peptide carboxyl group. Approximately 20 hydrogen bonds as well as van der Waals contacts involving approximately 80 to 100 atoms in 20 to 30 MHC side chains serve to bind a peptide in the cleft of a class I MHC molecule (9). Thus, the loss of a single hydrogen bond should represent a negligible loss in binding energy. Lys^{146} also appears to interact with the terminal carboxylate. A major difference between pockets A and F is the presence in the F pocket of the terminal NH_3^+ of Lys^{146} to neutralize the terminal carboxylate, whereas the A pocket does not contain a carboxylate to neutralize the terminal NH_3^+ of the nonapeptide directly (although a long range salt bridge to Glu^{63} is mediated through Tyr^7 and a water molecule) (9). This difference may also contribute to the functional importance of the hydrogen bonding network in the A pocket.

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Differential Display of Eukaryotic Messenger RNA by Means of the Polymerase Chain Reaction

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Effective methods are needed to identify and isolate those genes that are differentially expressed in various cells or under altered conditions. This report describes a method to separate and clone individual messenger RNAs (mRNAs) by means of the polymerase chain reaction. The key element is to use a set of oligonucleotide primers, one being anchored to the polyadenylate tail of a subset of mRNAs, the other being short and arbitrary in sequence so that it anneals at different positions relative to the first primer. The mRNA subpopulations defined by these primer pairs were amplified after reverse transcription and resolved on a DNA sequencing gel. When multiple primer sets were used, reproducible patterns of amplified complementary DNA fragments were obtained that showed strong dependence on sequence specificity of either primer.

Higher organisms contain about 100,000 different genes, of which only a small fraction, perhaps 15%, are expressed in any individual cell. It is the choice of which genes are expressed that determines all life processes—development and differentiation (1), homeostasis, response to insults, cell cycle regulation (2, 3), aging, and even programmed cell death. The course of normal development as well as the pathological changes that arise in diseases such as cancer (4), whether caused by a single gene mutation or a complex of multigene effects, are driven by changes in gene expression. Altered gene expression lies at the heart of the regulatory mechanisms that control cell biology. Comparisons of gene expression in different cell types provide the underlying information we need to analyze the biological processes that control our lives.

Current methods to distinguish mRNAs in comparative studies rely largely on the subtractive hybridization technique (5). A fingerprinting technique for mRNAs by two-dimensional (2-D) electrophoresis, such as has been used extensively in detecting cellular protein species (6), would be very useful. Reproducibility should be sufficient so that side-by-side comparisons of the mRNAs from different cells are possible. Furthermore, the identified spots should be usable for identifying and isolating the corresponding genes, mRNAs, or cDNAs. When protein gels were used frustration often followed because of the inability to obtain enough of the identified proteins for molecular characterization (7).

Our method is directed toward the identification of differentially expressed genes among the approximately 15,000 individual mRNA species in a pair of mammalian cell populations (8), and then recovering their cDNA and genomic clones. The general strategy is to amplify partial cDNA sequences from subsets of mRNAs by reverse transcription and the polymerase chain reaction (PCR). These short sequences are then displayed on a sequencing gel. Pairs of primers are selected so that each will amplify DNA from about 50 to 100 mRNAs because this number is optimal for display on the gel.

Selection of 3′ primers takes advantage of the polyadenylate [poly(A)] tail present on most eukaryotic mRNAs (9) to anchor the primer at the 3′ end of the mRNA, plus two additional 3′ bases. A primer such as 5′-T_{11}CA would allow anchored annealing to mRNAs containing TG located just upstream of their poly(A) tails (10). By probability this primer will recognize one-twelfth of the total mRNA population because there are 12 different combinations of the last two 3′ bases, omitting T as the

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penultimate base. The primer permits initiation of reverse transcription of only this subpopulation.

Any reverse transcribed cDNA species would be amplified by PCR if the distance at which a second primer anneals is smaller than 2 to 3 kb from the beginning of the poly(A) tail (an average molecular size of mRNA is 1.2 kb). Ideally this annealing position should be within 500 bp because cDNAs up to 500 bp can be resolved by size on a DNA sequencing gel. For a 5′ primer of arbitrary base sequence, annealing positions to cDNAs should be randomly distributed in distance from poly(A) tail. Therefore, the amplified products from various mRNAs will differ in size. After these PCR products have been labeled with [α-^{35}S]–labeled deoxyadenosine triphosphate (dATP), they would be displayed by autoradiography as a ladder on a sequencing gel.

The 5′ primer should in theory be short, 6 to 7 bp, for it to anneal fairly frequently near the end of a cDNA strand (Table 1). A critical technical problem is whether such short primers can give specific DNA amplification by PCR. Although arbitrary primers 8 to 10 nucleotides in length have been used for DNA polymorphism analysis by PCR (*11*), the standard PCR method uses primers of 20 or more nucleotides in length. After numerous trials with different primer sets and PCR conditions with cloned murine thymidine kinase (TK) cDNA (*12*) as a model template, the PCR parameters were chosen such that 42°C annealing would be optimal for product yield and specificity, whereas a 30-s elongation time would allow amplification of short products that could be resolved by a DNA sequencing gel. Primer $T_{11}CA$ in combination with a 10-mer (Ltk3) was found to give specific DNA amplification under these conditions (Fig. 1A). The specificity of DNA amplification dramatically increased with decreasing deoxynucleoside triphosphate (dNTP) concentration from that of standard conditions. Lowering the dNTP concentration to 2 μM not only improved the specificity of DNA amplification but also was necessary for labeling PCR products to a high enough specific activity with [α-^{35}S]-labeled dATP to provide high resolution on a DNA sequencing gel (Fig. 1C).

Primer-dependent DNA amplification was demonstrated because both primers were necessary for the specific amplification of the 301-bp TK cDNA fragment when either purified plasmid or lysate of *Escherichia coli* containing the plasmid was used as template (Fig. 1, B and C). The absence of bands in the *E. coli* genomic background demonstrates specificity of the reaction. Thus, a successful specific DNA amplification was achieved with a short primer set in combination with high resolution of the DNA sequencing gel.

Next, the method was applied to detect a subset of mRNAs in mammalian cells by comparing the TK message from quiescent versus cycling mouse A31 cells. Total RNAs (Fig. 2A) or mRNAs (Fig. 2B) were reverse transcribed with $T_{11}CA$ primer followed by PCR in the presence of the Ltk3 5′ primer. On the DNA sequencing gel, 50 to 100 amplified mRNAs ranging from 100 to 500 bp were visible, a number that seems to be optimal for analysis. Patterns of mRNA species seen between cycling and quiescent A31 cells (Fig. 2A) were very similar as expected, though specific differences were apparent. A band corresponding in size to the expected TK mRNA fragment was seen in the cycling cells but not in the quiescent cells obtained after serum starvation (Fig. 2A), which is consistent with TK mRNA production being G1/S phase specific (*13*). This experiment demonstrated that scarce mRNA species such as that of TK with copy numbers around 30 per cell can be detected (*13*). Repeated experiments produced highly reproducible patterns of cDNAs (95% bands were reproducible for a given pair of primers and mRNA sample in more than three independent experiments) (*14*). The reproducibility allowed direct comparisons between lanes (Fig. 2), further indicating the reliability of this method.

When normal (A31) and tumorigenic (BPA31) cells were compared, most bands were the same, but a few bands such as N1 were seen only in normal cells, or T1 in tumor cells (Fig. 2B). The band corresponding in size to the amplified TK mRNA fragment was clearly visible in both types of cells, representing a good internal control.

In accord with predictions, patterns of amplified cDNAs from human breast cancer and normal mammary epithelial cells were totally changed when either primer was changed (Fig. 3). Examination of these patterns provides further information regarding the differential display technique. When cDNA species were amplified with primer sets that differed by either the arbitrary primer (compare Fig. 3, lanes 1 and 2 with 3 and 4 and 5 and 6; lanes 7 and 8 with

Table 1. Theoretical calculation and experimental data of the number of mRNA species that can be amplified by arbitrary primers with different lengths in combination with an anchored oligo(dT) primer that binds to one-twelfth of the mRNA 3′ termini. The theoretical calculation is based on the estimation that a mammalian cell expresses about 15,000 different mRNA species (*8*) and that only amplified cDNA fragments with sizes smaller than 500 bp are visualized by a DNA sequencing gel.

Length of arbitrary primer (bases)	Kilobases per binding site	mRNA displayed (no.)	
		Theory	Experimental
6	4	150	0
7	16	38	0
8	65	10	0
9	262	2	20–30
10	1049	<1	50–100

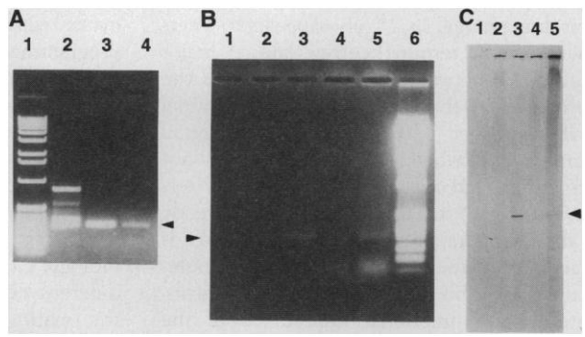

Fig. 1. Specific amplification of mouse TK cDNA template with primer set, $T_{11}CA$ and Ltk3 (CTTGATTGCC), which is located 278 bp upstream of its poly(A) tail (*12*). (**A**) A 1.5% agarose gel showing dNTP concentration dependence of the specificity of DNA amplification. The 10 ng of mouse TK cDNA plasmid pAMTK was amplified with the GeneAmp kit (Perkin-Elmer Cetus, Norwalk, Connecticut) in the presence of 2.5 μM $T_{11}CA$, 0.5 μM Ltk3 with dNTP concentrations at 200 μM (lane 2), 20 μM (lane 3), and 2 μM (lane 4). Other components in the PCR reaction were as suggested by the manufacturer. PCR parameters were 94°C for 30 s, 42°C for 1 min, and 72°C for 30 s with 40 cycles, and then 5-min elongation at 72°C. Lane 1 is the 1-kb ladder [Bethesda Research Laboratories (BRL), Bethesda, Maryland] as size markers. The arrowhead indicates the expected 301-bp amplified TK product. (**B**) A 1.5% agarose gel showing primer-dependent amplification of mouse TK cDNA template pAMTK in the *E. coli* genomic background, with the arrow indicating the amplified TK product. PCR conditions were as in (A) with 2 μM dNTP. Lanes 1 to 3 used 10 ng of pAMTK as template in the absence of $T_{11}CA$ (lane 1), in the absence of Ltk3 (lane 2), and in the presence of both primers (lane 3). Lanes 4 and 5 were in the presence of both primers but with lysates of *E. coli* LE392 lacking plasmid pAMTK (lane 4) and LE392 harboring about 30 copies of the plasmid per cell (lane 5) as templates, respectively. Bacterial lysates were prepared from single colonies on agar plates as described (*16*). Lane 6 is the 1-kb ladder. (**C**) A 6% DNA sequencing gel showing [α-^{35}S]dATP–labeled PCR product. Samples were as in (B) except 0.5 μM [α-^{35}S]dATP (1200 Ci/mmol) was included from the first cycle in the PCR reactions and 6.5 μl of sample was analyzed. The arrowhead indicates the amplified TK product.

9 and 10 and 11 and 12) or the anchored oligo-dT primer (compare lanes 1 and 2 with 7 and 8; lanes 3 and 4 with 9 and 10; lanes 5 and 6 with 11 and 12), they exhibited totally different patterns, verifying the rationale on which this method is based. The additional two bases in the anchored oligo-(dT) primers provided marked specificity, recognizing different but numerically similar subpopulations of the total mRNA. In general, each lane exhibited about 50 to 100 bands for the arbitrary 10-mers (G-C content of 50%); arbitrary 9-mers were less polymorphic. Although shorter arbitrary primers should by statistics recognize more mRNA species than longer ones (Table 1), primers with nine or fewer bases were actually poorer primers, as shown here and also by others (*11*). Hence, under these PCR conditions with Taq DNA polymerase used at its optimal temperature of 72°C, the shorter primers may have too low melting temperatures (below 40°C) to bind efficiently. Arbitrary 10-mers in conjunction with anchored oligo(dT) primers, can amplify more bands than statistics allows, in theory acting like 6- or 7-mers. This result suggests that they may hybridize to the target mRNA sequences in a degenerate fashion during the first few PCR cycles; this is supported by alterations in the 5′ sequences of their products. This degeneracy is advantageous in revealing an optimal number of mRNA species per gel. In theory, about 10,000 6- to 7-mer sequences are possible. The chance of finding any one such sequence in 500 bases thus is 0.05. Therefore 20 arbitrary 10-mers (priming as 6- to 7-mers) should statistically cover all mRNA sequences upstream of the 12 possible anchored oligo(dT) primers.

The next step was to recover cDNA of an identified mRNA species from a dried DNA sequencing gel and reamplify it with PCR. To obtain a probe, a DNA band from the sequencing gel was electroeluted with Hoefer's gel eluter and ethanol precipitated to remove contaminants such as urea. The yield of DNA recovery was typically 50%, measured by radioactivity. The recovered DNA was reamplified in the presence of 20 μM dNTP to achieve optimal yield and specificity. The reamplified PCR products were often visible on an agarose gel after two consecutive 40-cycle PCR reactions (*14*). This suggests that these short primers can amplify DNA but at the cost of low priming efficiency; therefore, more cycles or even rounds of PCR are necessary to produce enough DNA to be seen on an agarose gel. Reamplifications of N1 and T1 were shown to be dependent on both primers, and products corresponded with their sizes on the original sequencing gel (*14*).

As an internal control for specific recovery, a band corresponding to TK from tumorigenic cells (Fig. 2B) was recovered and reamplified. The reamplified product was characterized by both its correct total length and Stu I digestion to give two fragments of the expected sizes, one being 124 bp and the other 177 bp (*14*).

The reamplified N1 was cloned into plasmid pCR1000 and sequenced. The nucleotide sequence clearly shows that the N1 fragment is flanked by the mRNA mapping primer sequences of Ltk3 at the 5′ end and $T_{11}CA$ at the 3′ end as expected (Fig. 4A). DNA sequence comparisons between the arbitrary primers in the recovered cDNAs such as N1 and their corresponding original cDNAs isolated from cDNA libraries show that there were two to three mismatches at the 5′ end of the arbitrary primers (*14*). Searching the GenBank and EMBL DNA databases revealed that the partial N1 cDNA clone shows 70% identity in nucleotide sequence to an expressed sequence tag (EST00839) recently isolated by random sequencing of a human brain cDNA library (*15*).

Northern (RNA) blot analysis with the N1 probe detected a single mRNA species of about 3.5 kb. N1 mRNA appeared to be present at low abundance in both growing and quiescent normal cells. The blot confirms that N1 mRNA is present only in the normal A31 cells but not in the tumorigenic BPA31 cells (Fig. 4B). So far, a total of four cDNA sequences have been characterized. Of the three that were tested by Northern blot analysis, two including N1 turned out to be differentially expressed. The remaining one did not give any signal. It is possible that its mRNA message may be too low to be detected by Northern blot.

The differential display method described here is an alternative to subtractive or differential hybridization techniques. It can be used for three purposes. One is to visualize mRNA compositions of cells by displaying subsets of mRNAs as short cDNA bands; samples run in parallel reveal differences in their mRNA patterns. This is useful in the same way as 2-D protein gels, for example, to see and identify alterations in gene expression. Second, these cDNAs can be quickly sequenced; thereby a tag for each mRNA can be readily obtained and compared with sequences in data banks.

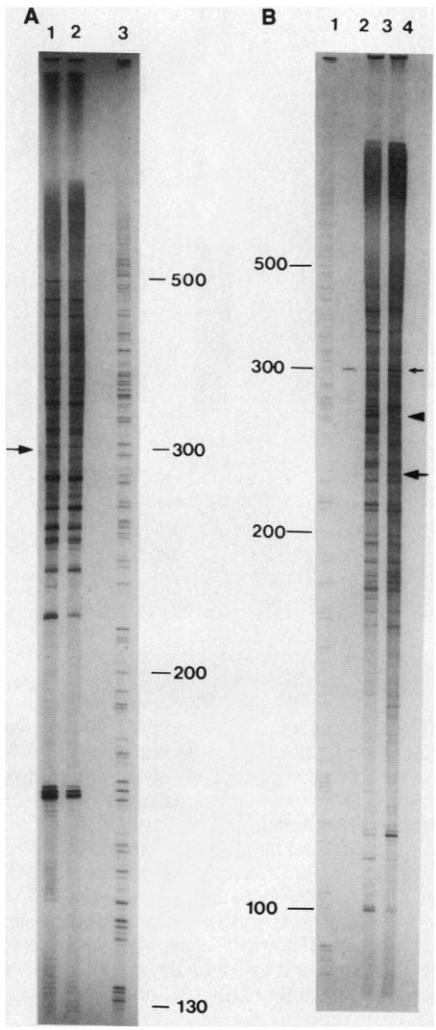

Fig. 2. Differential display using mRNA or total RNA. (**A**) Total RNA was isolated as described (*17*). Two micrograms of the total RNA from A31 grown in Dulbecco's minimum essential medium (DME) with 10% bovine calf serum until reaching 80% confluency (lane 1) or after 48 hours of 0.5% serum starvation (lane 2) were reverse-transcribed with 300 units of MMLV reverse transcriptase (BRL) in the presence of 2.5 μM of $T_{11}CA$ as primer and 20 μM dNTP for 60 min at 35°C. After heat inactivation of the reverse transcriptase at 95°C for 5 min, 2 μl of the sample was added to 18 μl of PCR labeling mix and amplified as in Fig. 2C. PCR product (6.5 μl) was analyzed on a 6% DNA sequencing gel. Lane 3, single-stranded DNA size markers of single track (ddG) DNA sequencing of human TMP kinase cDNA (*18*) with primer 5′-AAAAGCTTCTGAAGTTGTGGGGT. The arrow indicates amplified TK mRNA only present in the cycling cells (lane 1) but not in quiescent cells (lane 2). (**B**) Messenger RNA from 6×10^6 BALB/c 3T3 mouse fibroblast cell lines A31 (normal) and BPA31 (tumorigenic) growing in DME with 10% bovine calf serum and 10% CO_2 were isolated using the QuickPrep mRNA purification kit from Pharmacia–LKB Biochemical (Piscataway, New Jersey). The purified mRNA (0.5 μg) either from A31 (lane 3) or BPA31 (lane 4) were used as templates for the reverse transcription and subsequent PCR amplification as described above. Lane 1, ssDNA size markers of single track (ddC) DNA sequencing of human TMP kinase as described. Lane 2, 10 ng of pAMTK plasmid as template as external control. The small arrow indicates amplified TK mRNA as an internal control. The arrowhead indicates an amplified mRNA species (N1) seen only in normal A31 cell but not in the tumorigenic cell BPA31. The large arrow indicates an amplified mRNA species (T1) found only in the tumorigenic BPA31 cells.

Third, individual bands can readily be cloned and used as probes for Northern or Southern (DNA) blottings and to isolate genes from cDNA or genomic libraries.

Differential display has several technical advantages as compared to subtractive and differential hybridizations. It is much quicker; 2 months are required to isolate clones from cells by subtractive hybridization, which includes mRNA isolations, cDNA library constructions, subtraction, and screening by differential hybridization. With differential display the band patterns are obtained in 2 days and clones in 5 days. In addition, unlike subtractive hybridization, differential display allows simultaneous detections of both groups of differentially expressed genes (for example, candidate tumor suppressor genes and oncogenes). Most genes by statistics should be present in the patterns as single bands. Therefore, redundancy, underrepresentation of rare mRNAs, and false positive clones are minimized. In terms of sensitivity, because the method described here is PCR-based, only 1 μg of mRNA is required per 100 lanes, compared to 50 times as much or more for subtractive hybridization. A direct comparison of the number of cDNA bands amplified by a given set of primers with either a cDNA library or mRNA of the same cell type indicates that the new method is much more sensitive (*14*). This suggests possible underrepresentations of many genes during cDNA library constructions. Reproducibility from run to run of the method in displaying mRNA patterns with the same RNA sample is high (>95% bands are always seen) in comparison with the great variations in the kinds and numbers of genes isolated by each subtractive hybridization. The advantage of subtractive hybridization is its enrichment and focus on only the differentially expressed genes. Because of its simplicity based on PCR and a DNA sequencing gel, two of the most widely used molecular biological techniques, the differential display technique should find wide and rapid applications in studying cancer, heart disease, cell differentiation, and aging, among others.

These results demonstrate the potential of this technique to identify differentially expressed mRNAs and to clone their genes. It should make possible the detection of most of the mRNAs in a cell by use of multiple primer sets.

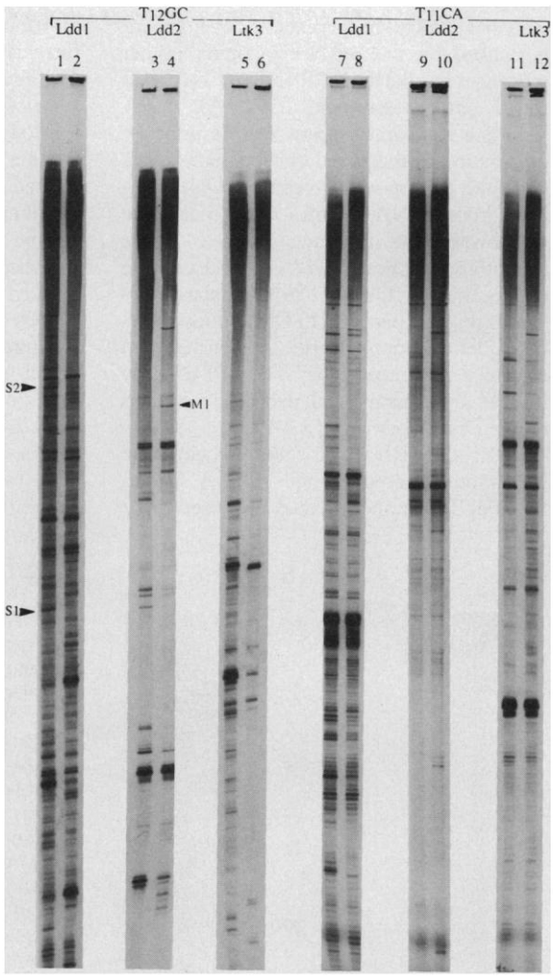

Fig. 3. Differential display of mRNAs from a normal versus a metastatic human breast cancer cell using multiple primer sets. Both normal (76N) and tumor (21MT-2) cells were cultured in D medium (*19*) until reaching about 70% confluency before the polyadenylated RNAs were extracted using the Quickprep mRNA purification kit from Pharmacia–LKB Biochemical. The purified mRNA (0.5 μg) was reverse transcribed with either $T_{11}CA$ or $T_{12}GC$. Six different combinations of primer sets made of two anchored oligo-dT primers ($T_{11}CA$: 5′-TTTTTTTTTTTCA-3′ and $T_{12}GC$: 5′-TTTTTTTTTTTGC-3′) and three short arbitrary primers (two are 10-mers, Ldd1: 5′-CTGATC-CATG-3′ and Ltk3: 5′-CTTGAT-TGCC-3′, and one is a 9-mer, Ldd2: 5′-CTGCTCTCA-3′) were used for the PCR reactions essentially as described, except the annealing temperature was at 40°C instead of 42°C. The odd-numbered lanes correspond to mRNA from normal cells whereas even-numbered lanes represent mRNA from the tumor cells. Several candidate cDNA tags that appear to be differentially expressed are marked by arrowheads.

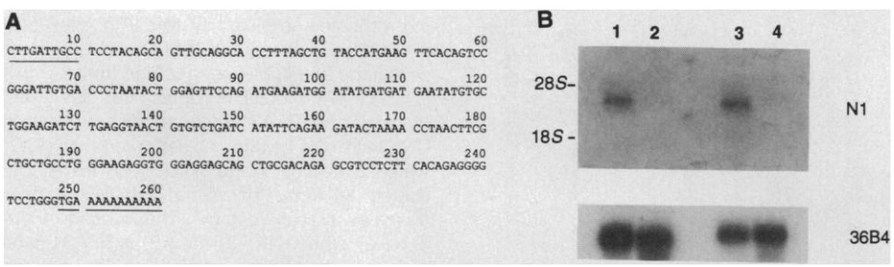

Fig. 4. (**A**) Nucleotide sequence of N1. Reamplified N1 fragment was cloned into pCR1000 to give pCR-N1 with the TA cloning system from Invitrogen (San Diego, California). Both strands of N1 were sequenced with the M13-40 and T7 promoter primers. Nucleotide sequences of flanking mRNA mapping primers are underlined. (**B**) Northern blot analysis of N1 mRNA in normal and tumorigenic mouse fibroblast cell lines. Cellular total RNA extraction and Northern blot were carried out as previously described (*20*). N1 was gel purified as an Eco RI–Hind III fragment from pCR-N1 and labeled with [α-^{32}P]dCTP to a specific activity of 1×10^9 cpm/μg of DNA with a random-prime DNA labeling kit from Boehringer Mannheim Biochemicals (Indianapolis, Indiana). A sample (20 μg) of total RNAs from either exponentially growing normal A31 cells (lane 1) and tumorigenic BPA31 cells (lane 2) or quiescent normal A31 cells (lane 3) and tumorigenic BPA31 cells (lane 4) obtained after 45 hours of serum starvation were analyzed. The blot was exposed for 72 hours with intensifying screens. As a loading control, the same blot was reprobed with 36B4 cDNA (*21*) and exposed for 16 hours without screen.

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16. Single bacterial colonies were transferred with sterile toothpicks into 50 μl of TE buffer containing 0.1% Tween 20. After incubating at 95°C for 10 min, cell debris was pelleted by centrifugation for 5 min in an Eppendorf microcentrifuge. The supernatant was used as source of DNA for the PCR.
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Biosynthesis of Human Papillomavirus from a Continuous Cell Line Upon Epithelial Differentiation

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The study of the human pathogen papillomaviruses (HPVs) has been hampered by the inability to propagate the virus in tissue culture. The addition of 12-*O*-tetradecanoyl phorbol-13-acetate to the media of organotypic (raft) cultures increased expression of physiological markers of keratinocyte differentiation and concomitantly induced production of virions. Capsid production was detected in differentiated suprabasal cells. Virions approximately 54 nanometers in size were observed by electron microscopy in raft tissue cross sections in the suprabasal layers. Virions purified through isopycnic gradients were found to contain type 31b DNA and exhibited an icosahedral shape similar to that of papillomaviruses found in clinical samples.

Human papillomaviruses (HPVs) are important human pathogens associated with a variety of neoplasias. HPV types 16, 18, 31, 33, 35, and 51 have been associated with malignant lesions of the anogenital area, and types 6 and 11 are found in benign genital lesions (*1*, *2*). Study of the complete viral life cycle has been prevented by the lack of a cell culture system that will permit vegetative viral replication. Researchers have propagated HPVs in rodents by grafting infected tissue either under the renal capsule (*3*) or under the flank skin (*4*) of a nude mouse, but no reproducible permissive in vitro system has yet been described. This is probably a result of the evolution of a viral life cycle that is tightly coupled to the differentiation program of keratinocytes in which virion production is limited to differentiating suprabasal cells (*5*).

Organotypic (raft) cultures recreate important features, both morphological and physiological, of epithelial differentiation in vitro by raising the cells to an air-liquid interface. This has been accomplished by the recombination of epidermal cells with a collagen matrix maintained on rigid support (*6*). However, raft cultures have continued to be deficient in the most important aspect of papillomavirus research, the ability to propagate virions. One stage of productive infection, the differentiation-specific amplification of episomal viral DNA in the upper layers of the epithelium, has been achieved in raft cultures (*7*). This was done with a cell line (CIN-612) derived from a cervical intraepithelial neoplasia type 1 (CIN 1) lesion that maintains episomal copies of HPV type 31b DNA. No evidence of virion production was detected with this cell line. Although important for replication studies, the ultimate goal of complete vegetative viral replication in vitro was still lacking.

One explanation for this inability to propagate HPVs in culture was the failure of the raft system to faithfully duplicate all aspects of the differentiation program of epithelial cells to which viral production is closely linked (*8*, *9*). In normal keratinocytes, keratin 10 is expressed throughout the suprabasal layers of the differentiating epithelium (*9*, *10*), and filaggrin is generally restricted to the stratum granulosum of the differentiating epithelium (*11*). In raft cultures of CIN-612 cells, keratin 10 (Fig. 1C) was only weakly present in the intermediate layers, and filaggrin was only weakly expressed in the stratum corneum and occasionally in the upper part of the stratum granulosum (Fig. 1E). When 12-O-tetradecanoyl phorbol-13-acetate (TPA) was added to the raft culture media of invasive cervical carcinoma cell lines (*12*), morphological differentiation was induced, as shown by cross sections of raft cultures (*13*). In TPA-treated CIN-612 rafts, keratin 10 was ex-

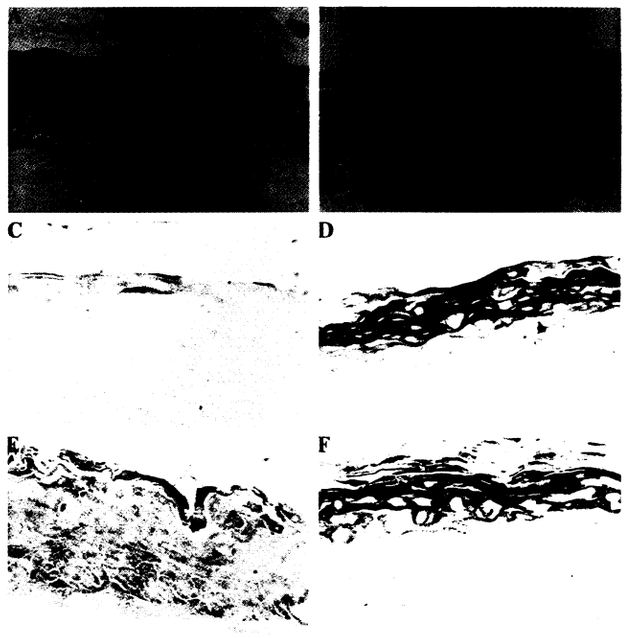

Fig. 1. Effect of TPA treatment on the expression of epithelial differentiation-specific markers in CIN 1–derived CIN-612 raft cultures. We examined the expression of keratin 10 and filaggrin by immunostaining thin sections of paraformaldehyde-fixed, paraffin-embedded raft tissue with the VECTASTAIN Elite ABC Kit (Vector Laboratories). (**A** and **B**) CIN-612 raft cultures with haematoxylin and eosin staining. (**C** and **D**) CIN-612 raft cultures immunostained with a keratin 10–specific monoclonal antibody (Accurate Chemical & Scientific Corp.) as the primary antibody. (**E** and **F**) Immunostaining of CIN-612 raft cultures with a filaggrin-specific monoclonal antibody (Biomedical Technologies Inc.) as the primary antibody. In (A), (C), and (E), CIN-612 raft cultures were grown as described (*24*) without TPA in the media. In (B), (D), and (F), CIN-612 raft cultures were treated with 16 nM of TPA for 16 to 24 hours every fourth day for 16 days.

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