# Characteristics of Oligonucleotide Uptake in Human Keratinocyte Cultures

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Oligodeoxyribonucleotides have the potential to interfere selectively with cellular protein synthesis by sequence-specific hybridization to DNA or RNA molecules. We have investigated the properties of uptake and intracellular localization of fluorescently labeled oligonucleotides in cultured human keratinocytes using confocal laser scanning microscopy. Unlike many other cell types studied, keratinocytes can internalize oligonucleotides without apparent sequestration in endosomes or cell surface accumulation. Uptake is primarily nuclear and unaltered by sodium azide, monensin, or chloroquin pretreatment. We have verified our results with two different fluorophores, fluorescein and Bodipy, and found similar uptake and distribution patterns in both live and fixed cell populations. Surprisingly, we have found uptake to be heterogeneous within a population, with 15–30% of cells internalizing the oligonucleotides. This percentage is drastically increased to roughly 80% at cell population margins, and after release from M phase arrest. These results on uptake and intracellular localization suggest that keratinocytes may have increased sensitivity as target cells for oligonucleotide based gene regulation strategies. Key words: antisense/triplex. *J Invest Dermatol 101:727–731, 1993*

Oligodeoxyribonucleotides (ONs) are becoming increasingly popular as mediators of selective inhibition of gene expression. These short single-stranded DNA molecules can be designed to hybridize locally either with cellular DNA (triplex ONs) or RNA (antisense ONs) and interfere with subsequent transcription or translation, respectively [1–6]. Ever since the first report of antisense ON mediated gene suppression over a decade ago [4], the number of successful antisense citations has been increasing exponentially. More recently, triplex ONs have been described that also selectively inhibit protein synthesis [5,6]. The most common gene targets of antisense and triplex approaches are viral proteins involved in replication and pathogenicity [7,8], cellular oncogenes implicated in neoplastic transformations [9–11], and specific cellular proteins involved in disease pathophysiology [12,13].

Antisense and triplex technology may have particular application in the dermatologic sciences owing both to the multitude of potential disease states, i.e., infectious (Herpes), proliferative (psoriasis), invasive (carcinoma), as well as the potential ease of site-specific delivery. Several reports have already emerged citing the ability of a sequence-specific ON to decrease c-myc levels and subsequent proliferation in keratinocytes [14] and mouse epidermis [15]. In fact, the first antisense clinical trials involved the topical application of an anti-Herpes ON. Research is also currently exploring the feasibility of iontophoretic approaches to delivery of ONs.

Regardless of application or site, the introduction of an ON into the cell where it can exert its activity remains a significant hurdle. Despite a growing research base, considerable debate still exists on the mechanisms of uptake, extent of uptake, and intracellular localization of ONs that are incubated with different cell populations [16–19].

We used confocal laser scanning microscopy to investigate whether the rate and extent of ON uptake was similar in various cell types, to determine if primary cells internalized ON to the same extent as corresponding immortalized cells, and to determine the intracellular fate of internalized ONs. This technique has several distinct advantages over tracking radiolabeled ONs by subcellular fractionation and autoradiography. Not only can one definitively distinguish between cell surface–associated and intracellular ON and localize the ON subcellularly, but also one is able to observe cell-to-cell heterogeneity in uptake properties rather than obtain data as an average of a large population.

Our studies indicate that all cell types do not internalize ON to the same extent or by the same mechanism. Human cultured primary keratinocytes appear to have unique properties of ON uptake and intranuclear localization quite distinct from a variety of other cell types, including the immortalized HaCat keratinocyte cell line. Such observations may have important implications for dermatologic applications of antisense- and triplex-based gene regulation strategies.

## MATERIALS AND METHODS

**Preparation of Keratinocytes** Second-passage keratinocytes were obtained from neonatal human foreskins using 0.25% trypsin and grown in keratinocyte growth medium (KGM) (Clonetics, San Diego, CA), containing 0.07 mM calcium. Cells were plated onto 12-mm coverslips within 24-well plates or in 4-well chamber slides (Nunc, Naperville, IL) and maintained at 37°C, 5% $CO_2$. Where noted, keratinocyte proliferation was halted at 40% confluence by demecolcine (Sigma, St. Louis, MO) for 12 h at a concentration of 0.05 μg/ml as previously described [20]. Cells were released by washing twice in phosphate-buffered saline (PBS) with subsequent addition of fresh media, and allowed to settle for 2 h before ON incubation. Where noted, keratinocytes were pretreated with 10 mM so-

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Abbreviation: ON, oligodeoxyribonucleotide.

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dium azide, 0.1 mM monensin, or 0.1 mM chloroquin (Sigma, St. Louis, MO) for 15 or 30 min.

**Fluorescent Oligonucleotides** Two different fluorescently labeled 26-mer ONs were synthesized (Keystone Laboratories, Menlo Park, CA). The first was a 26-nucleotide sequence composed of guanine and thymine with its 3′ end conjugated to Bodipy (Molecular Probes, Eugene, OR). The second also contained only guanine and thymine but had fluorescein phosphoamidites (Clontech, Palo Alto, CA) conjugated at base positions 4, 13 (where noted), and 23, and had $CH_2CH(OH)CH_2NH_2$ conjugated to its 3′ hydroxyl group for exonuclease protection. All ONs were high-performance liquid chromatography purified by Keystone Labs using a Hamilton PRP-1 reverse-phase column. Fluorescently labeled ONs were added at a concentration of 20 $\mu$M and incubation proceeded for 2 h at 37°C.

HeLa, MCF-7, and HaCat cells were treated as above, but grown in different media conditions. HeLa cells were grown in RPMI-1640 without phenol red, MCF-7 cells were grown in Dulbecco's modified Eagle's medium without phenol red and supplemented with 7.8 $\mu$g/ml insulin, and HaCat cells were grown in Dulbecco's minimum essential medium. Each medium contained 10% heat-inactivated fetal bovine serum and was supplemented with penicillin and streptomycin at 100 u/ml (Gibco, Grand Island, NY).

**Preparation of Coverslips and Slides** After incubation, cells were viewed by confocal microscopy as either live or fixed specimens. Live specimens were prepared by washing coverslips in PBS and mounting on a chamber containing 200 $\mu$l of PBS as described previously [21]. Fixed specimens were prepared by washing coverslips or chamber slides in PBS and immersing in 3.7% paraformaldehyde for 20 min at 25°C. Fixed specimens were viewed within 6 h of fixation.

**Confocal Laser Scanning Microscopy** Microscopy was performed on a Biorad MRC600 (Cambridge, MA) confocal laser scanning microscopy system equipped with an argon ion laser exciting maximally at 488 nm and 514 nm and operating under CoMoS software. Blue high-sensitivity filter blocks contained excitor filter 488 nm DF 10, dichroic reflector DR 510 nm LP, barrier emission filter OG = 515 nm. The confocal scanning system was mounted on an upright Nikon Optiphot microscope containing a 20× Zeiss objective, NA = 8 and a 60× Nikon Planapo objective, NA = 1.4.

All images are generated from a frame of 512 × 768 pixels with a laser dwell time of 2.54 $\mu$seconds/pixel. Neutral density filters and apertures were set to minimize cellular phototoxicity and autofluorescence. Photon-counting mode was used for quantitative data analysis and, in all images, the enhance function was set to the "off" position to maintain linearity between input photons and output voltage.

## RESULTS

We have used confocal laser scanning microscopy to study fluorescently labeled ON internalization in a variety of primary cells and cell lines, and have found cultured human keratinocytes to be unique in their uptake and localization properties (Figs 1 and 2). All cells shown were incubated with an internally labeled fluorescein ON or 3′ Bodipy end-labeled ON at 20 $\mu$M concentration at 37°C, 5% $CO_2$ for 2 h. Cells in Figs 1 and 2*a–c* were viewed live while surrounded by buffered saline to prolong cell health and minimize local pH fluctuations and autofluorescent artifacts. Viability was verified by propidium iodide dye exclusion in double-labeling parallel experiments.

HeLa cells (human cervical carcinoma, Fig 1*a*), MCF-7 cells (human breast carcinoma, Fig 1*b*), as well as several other nonepithelial cell lines such as leukemic HL60 cells [16] and L929 fibroblasts [17] tend to accumulate fluorescent ON on their cell surface with smaller amounts found concentrated in punctate vesicles intracellularly. Uptake in these cells is strongly temperature dependent, with greatly reduced cell surface association and internalization seen at lower temperatures of 25°C and 4°C [16,18,19].

In sharp contradistinction, cultured primary keratinocytes appear to internalize far greater amounts of ON and to concentrate the ON intranuclearly (Fig 2) rather than in endosome-like structures or on the cell surface. In addition, uptake and localization were not significantly altered by incubation at the lower temperature of 25°C, nor by pretreatment with the active transport inhibitors sodium azide, monensin, or chloroquin for 15 or 30 min.

Interestingly, when identical experiments were carried out in the spontaneously immortalized HaCat keratinocyte cell line (Fig 1*c*), results were consistent with those seen in HeLa, HL60, and MCF-7 cells. Fluorescence was markedly punctate, cytoplasmic, and significantly reduced at the lower temperature of 4°C.

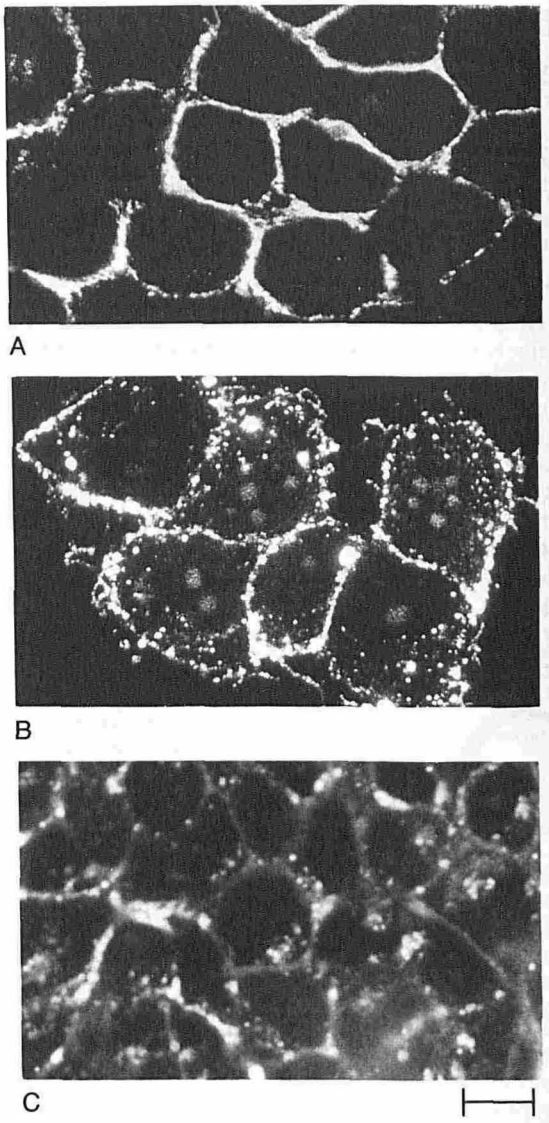


**Figure 1.** Oligonucleotide uptake in HeLa *(A)*, MCF-7 *(B)*, and HaCat cells *(C)*. All cells were incubated with 20 $\mu$M of fluorescein labeled oligonucleotide for 2 h and viewed live with a 60× objective lens and 3% laser power. *Scale bar*, 10.2 $\mu$M *(A)*, 12.5 $\mu$M *(B)*, 13.2 $\mu$M *(C)*.

To reduce the possibility that uptake and localization were driven by the properties of the fluorophore rather than the ON, we performed keratinocyte experiments with two fluorophores having distinct chemical properties. Whereas fluorescein is a pH-sensitive polar molecule, Bodipy is a nonpolar lipophilic pH-insensitive molecule. Despite the chemical and structural differences of the fluorophores themselves, intracellular uptake and intranuclear localization features were maintained (Figs 2*a–c*). We also performed experiments with ONs of identical length and fluorescein placement, but composed equally of adenine, guanine, cytosine, and thymine bases, and ONs composed mainly of guanine residues. No substantial differences in uptake or distribution were noted with changes in sequence, composition, or relative G content. Intracellular localization was also unaltered by keratinocyte incubation with 8-mer guanine/thymine-rich ONs internally labeled with fluorescein (images not shown).

We performed quantitative photon counting of a population of

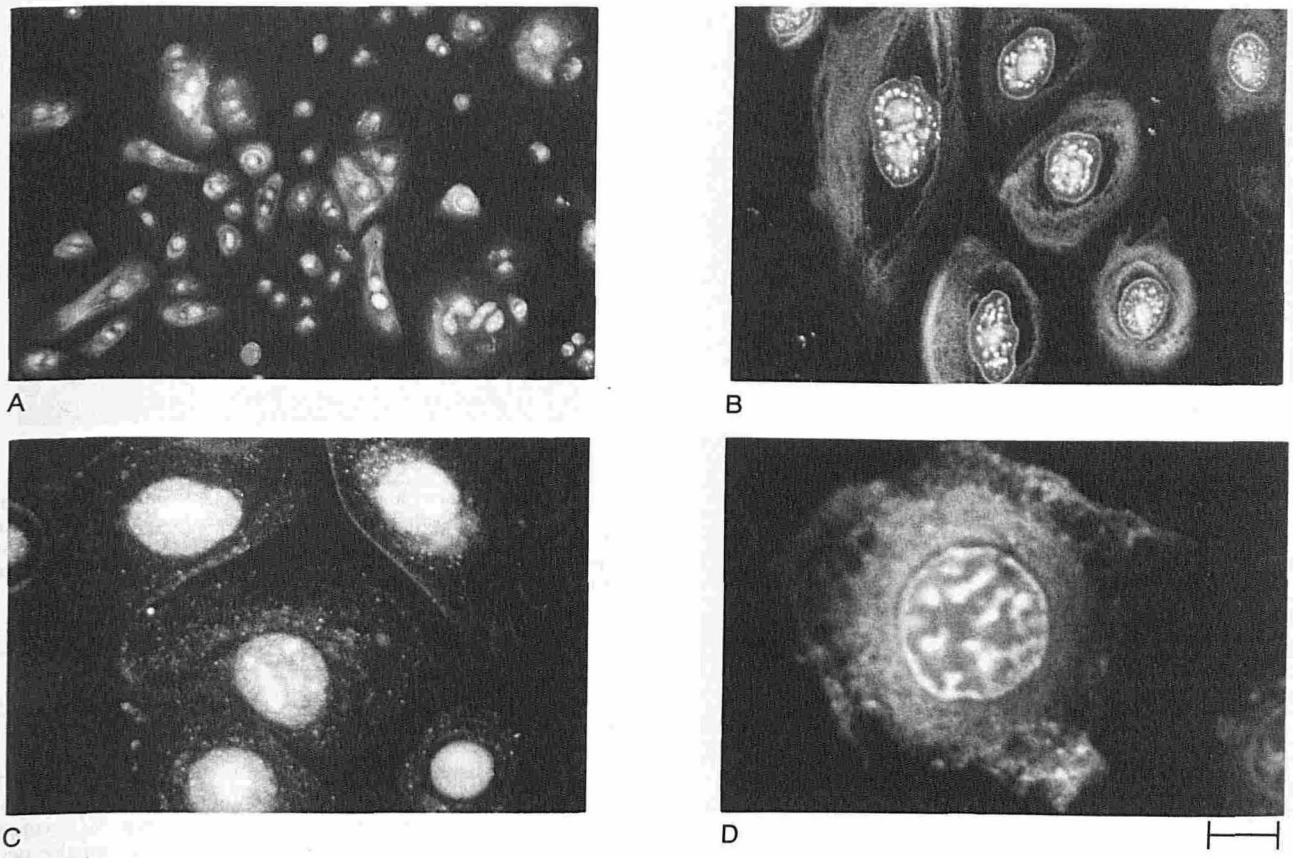


**Figure 2.** Oligonucleotide uptake in human cultured keratinocytes. Keratinocytes were incubated with 20 μM fluorescein *(A,B,D)* or Bodipy *(C)*-conjugated oligonucleotide for 2 h. *A)* Keratinocytes viewed live with 20× objective, 3% laser power. Fluorescein was conjugated at base positions 4, 13, and 23. *Scale bar,* 54.5 μM. *B)* Same keratinocyte population as in *A* viewed live with 60× objective, 3% laser power. *Scale bar,* 15.9 μM. *C)* Keratinocytes viewed live with 60× objective and additional 1.9× optical magnification, 1% laser power. *Scale bar,* 10.4 μM. *D)* Fixed keratinocytes viewed with 60× objective and 2.0× optical magnification. Fluorescein was conjugated at base positions 4 and 23. *Scale bar,* 6.25 μM.

cells internalizing the pH-insensitive Bodipy ON and found the nuclear : cytoplasmic fluorescence ratio of representative cell cross-sectional areas to average 2.5 : 1. This value was derived by averaging all pixels within the nuclear compartment of each cell and dividing by the average of all pixels within the cytoplasmic compartment. Laser and aperture settings were unchanged during image acquisition to ensure constant image thickness.

As confocal microscopy in live cells can introduce artifacts from autofluorescence and phototoxicity, we have also confirmed our results on uptake and localization in fixed keratinocytes. Figure 2*d* demonstrates keratinocytes that have been incubated with 20 μM fluorescein ON for 2 h, followed by washing in PBS and fixation in 3.7% paraformaldehyde. As this crosslinking fixative can be reversible, cells were viewed within 6 h of fixation. Similar results were found with the Bodipy-labeled ON in fixed cells.

To reduce the possibility that the observed uptake patterns were due to impure ON samples containing unconjugated fluorophores, the samples were purified by high-performance liquid chromatography before cell incubation. To reduce the possibility that the observed uptake fluorescence was a result of intracellular or extracellular nuclease degradation and fluorophore release, we examined the pattern of uptake in the presence of unconjugated fluorophore. Figure 3*a* and *b* demonstrates the fluorescence obtained when keratinocytes were incubated with free fluorescein and Bodipy, respectively. We also performed the experiment with a fluorescein phosphoramidite conjugated to a single guanine nucleotide with a 3′ amine modification. Cellular fluorescence obtained closely resembled Fig 3*a*, providing further evidence that Fig 2*a*, *b*, and *d* is not the result of completely metabolized ON.

Another noteworthy distinction between ON uptake in cultured keratinocytes versus other cell types studied is the lack of homogeniety in uptake (Fig 4*a,b*). In HeLa, MCF-7, and HaCat cells, all viable cells appear to associate with and internalize ON to roughly the same extent. However, in keratinocytes, ON uptake is extremely heterogeneous within a single cell population. Uptake within a population of confluent cells ranges from 15–30%, with the majority of these cells localized at population borders. However, when cells are halted in M phase for 12 h by the stathmokinetic agent demecolcine, released, and then incubated with fluorescent ON for 2 h, the percentage of cells internalizing ON increases to nearly 80%. The patterns and distribution of fluorescence, however, are unchanged.

## DISCUSSION

In this report we present evidence of ON uptake by human cultured keratinocytes. Confocal laser scanning microscopy establishes the intracellular localization of fluorescein or Bodipy-labeled ONs to be primarily nuclear, with increased intensity at nucleolar regions. We do not observe in primary keratinocytes the more characteristic punctate intracytoplasmic fluorescence that has previously been attributed to endosomal localization [16,17,19].

The use of fluorophores with different chemical properties

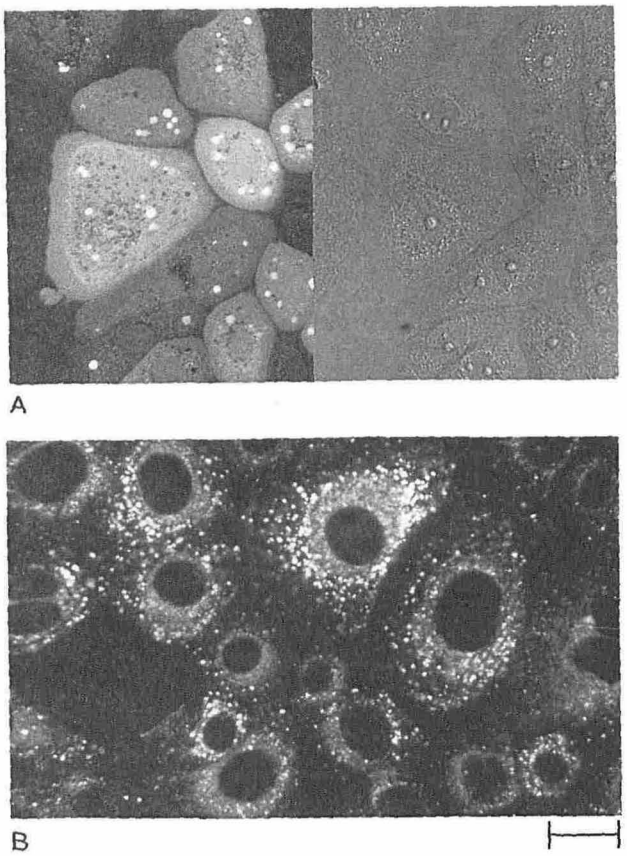


**Figure 3.** Free fluorophore uptake in human cultured keratinocytes. Keratinocytes were incubated with 60 μM free fluorescein *(A)* or Bodipy *(B)* for 2 h and viewed live with 60× objective. *A* had additional 1.8× optical magnification and 3% laser power; *scale bar,* 20.1 μM. *B* was obtained with 1% laser power; *scale bar,* 20.6 μM.

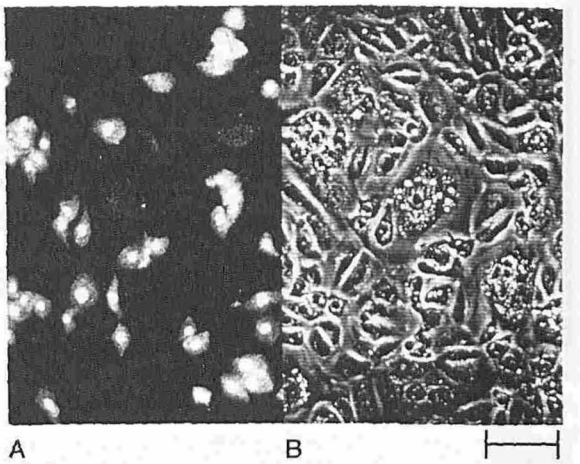


**Figure 4.** Heterogeneity in oligonucleotide uptake. Simultaneous fluorescence *(A)* and phase-contrast *(B)* image of keratinocytes demonstrating cell-to-cell heterogeneity in uptake of a fluorescein labeled oligonucleotide (base positions 4, 13, 23) after a 2-h incubation. Image was obtained with 20× objective and 3% laser power with live cells; *scale bar,* 60.6 μM.

allows for the corroboration of uptake properties. For instance, in fluorescein ON images of live cells, a dark perinuclear region is observable that is not found in fixed preparations or with Bodipy ON live images. As fluorescein is strongly pH sensitive between the ranges of 5 and 9, this perinuclear dark region presumably represents acidic compartments. In fixed cells the pH gradient is lost, and in live Bodipy images the fluorescence is not pH sensitive. This interpretation is consistent with the location of acidic compartments in keratinocytes grown in low-calcium conditions [22].

Two interesting questions arise from our investigations. First, why are primary human keratinocytes unique in their uptake and intracellular localization properties? Second, what is the basis for the heterogeneity in uptake among a given cell population? Our current research is aimed at resolving these questions, and we are investigating several possibilities.

As for the first question, the unique uptake and localization of fluorescent ONs in keratinocytes could conceivably arise in one of two ways—by an endocytotic pathway with leakage before metabolic breakdown, or by a nonendocytic predominantly passive process. We are currently in favor of the latter hypothesis, as previous time-course studies after 5-, 15-, and 60-min incubations do not demonstrate any additional evidence of endocytic fluorescence, and uptake is not inhibited by 25°C incubation nor by pretreatment with sodium azide, chloroquin, or monensin. In addition, microinjected free cytoplasmic ONs have been demonstrated to accumulate rapidly in the nucleus [23,24] whereas endosomal ONs remain primarily in punctate vesicles. Because keratinocytes accumulate ON intranuclearly and not within vesicles, we hypothesize that the process is not mediated by either receptor-mediated or fluid-phase endocytosis.

The issue of heterogeneity in uptake is equally enigmatic. Heterogeneity has previously been described in lymphoid cell populations [25], but its basis in these cells is also unknown. We are presently considering three possible explanations—that uptake occurs mainly in a subset of proliferating cells, that uptake occurs in a cell-cycle specific fashion, or that uptake occurs as an early feature of programmed cell death before morphologic membrane disruption is detectable.

Evidence for the first hypothesis includes observations of increased uptake at population margins and within subconfluent regions, observations of decreased uptake when incubation takes place at 100% confluence, and previous reports of the existence of various keratinocyte subpopulations with greatly varying generation times [20,26–28]. Further research with synchronized keratinocyte populations and with fluorescent antibody markers of programmed cell death may help determine the basis for heterogeneity.

The nuclear concentration of ON has several implications for dermatologic applications of antisense technology. Nuclear splice sites, branch points, and 5′ cap sites of premature mRNA may provide better target sites than cytoplasmic translation start codons. Antisense activity may occur at lower ON concentrations in keratinocytes than in other cell types. In addition, keratinocytes may represent a good experimental cell type for recently described triple-helix–based strategies of gene suppression [5,6] that require nuclear localization of ON. However, our studies do not establish the concentration of intracellular ON in free (unbound) form, which presumably represents the biologically active fraction.

To conclude, we find human cultured keratinocytes to be unique among other cell types in their ON uptake and intracellular localization properties. Ongoing studies comparing the biologic activity and dose response of ONs should provide increased insight into the applicability of antisense and triplex ONs in dermatologic research.

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