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Binding of Fluorescent Dye to Genomic RNA Inside Intact Human Rhinovirus after Viral Capsid Penetration Investigated by Capillary Electrophoresis

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RiboGreen is used for concentration measurements of RNA. Upon binding to the RNA, an ∼1000-fold increase in sensitivity in comparison with the UV absorbance of the free polynucleotide is observed. In the present work, we demonstrate that this dye can penetrate in a time- and temperature-dependent manner the intact viral capsids of human rhinovirus serotypes 2 and 14, where it forms a fluorescent complex with the viral RNA. Capillary electrophoresis with laser-induced fluorescence detection of virus incubated with RiboGreen shows that the electrophoretic mobility of the viruses remained unchanged upon dye-binding. As shown for human rhinovirus serotype 2, its native conformation was conserved, since it still bound a recombinant soluble receptor fragment derived from the very low density lipoprotein receptor. The labeled RNA was released by heat-induced uncoating of the virus, and the RNA−dye complex could be directly detected if degradation was prevented with an RNase inhibitor. This in vitro labeling of viral RNA encased within a protein shell demonstrates the virion's dynamic nature that temporarily allows access of a low-molecular-mass compound to the otherwise protected RNA. It might be of great value for experiments requiring fluorescent viral particles with an unmodified surface, such as investigations of endocytosis and viral uncoating on the single molecule level.

Human rhinoviruses (HRVs) belong to the genus *Picornaviridae* and share capsid architecture and genome organization with a number of serious human and animal pathogens, such as polio or foot-and-mouth disease virus. HRVs are the main causative agents of the less serious but bothersome common cold.[1] Their icosahedral capsid of ∼30 nm in diameter is composed of four viral proteins (VP1, VP2, VP3, and VP4), with the first three being exposed at the surface and VP4 being internal and in intimate contact with the 7100-bp-long single-stranded messenger sense RNA genome. The minor group of HRVs comprises 10 serotypes, with the prototype strain HRV2, which all bind members of the low-density lipoprotein receptor (LDLR) family such as LDLR, very-LDLR, and LDLR related protein (LRP).[2,3] The major group comprises 91 serotypes, which bind intercellular adhesion molecule 1 (ICAM-1).[4] Because virus particles possess a ζ-potential, they migrate electrophoretically under the influence of an electric field as other submicrometer- or micrometer-sized particles, such as organelles, bacteria, or cells (see, e.g., refs 5−10).

Native virions sediment at 150S in sucrose density gradients, but subviral particles sedimenting at 135S and at 80S also exist. These include precursors of the native virus, being produced during assembly, and intermediates of the uncoating process, which results in the release of the RNA into the cytosol of the host cell, initiating infection.[11−13] "A" (135S) particles have lost the innermost capsid protein VP4; "B" (80S) particles have, in addition, also lost the RNA. Both subviral particles no more bind to the viral receptor proteins.

In the case of poliovirus, it was observed that antibodies directed against peptides derived from parts of the proteins in the interior of the capsid were able to attach to the virus upon incubation at 37 °C.[14] This was interpreted as an indication that the virion was highly dynamic and that these sequences became temporarily exposed at physiologic temperature, a process termed "breathing". The dynamic nature of the virus capsid was also confirmed by tryptic digestion of HRV14, followed by mass spectrometric determination of the released peptides.[15,16] In these

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studies, it was demonstrated that capsids' binding antivirally active drugs, such as the WIN compounds (for Sterling Winthrop, the company, which produced them), strongly reduced the appearance of peptidic cleavage products. This indicates that the compounds indeed inhibit the movements of the polypeptide chains and thereby stabilize the capsid.[17] By binding within a hydrophobic pocket, they occupy the space required for displacement of the peptide chains.[18] Using capillary electrophoretic methods, we recently also demonstrated that thermal decomposition of the virus is slowed in the presence of WIN compounds. In another study, Broo and colleagues showed that the target of various derivatives of aziridine, as used for inactivation of viruses in vaccine manufacturing, was not the protein, but rather, the nucleic acid.[19] The authors of this paper demonstrated that the compound gained access to the nucleic acid through the intact capsid of HRV14 and other viruses, presumably because of the dynamic nature of the virion.

Aiming at the production of fluorescence-labeled HRV2, we reasoned that RiboGreen, a fluorescent dye (ex/em: 500/525 nm, bound to nucleic acid) commercially available for RNA concentration determination might also be able to penetrate the viral capsid without changing its surface architecture. Using the highly sensitive technique of laser-induced fluorescence (LIF) detection in conjunction with capillary electrophoresis (CE), we investigated whether this is, indeed, the case. We demonstrate that the penetration of the dye is temperature- and time-dependent and that the fluorescence-labeled virus is still in a native conformation.

EXPERIMENTAL SECTION

Instrumentation. Capillary electrophoresis with fluorescence detection was carried out with a homemade instrument equipped with a LIF detector (Ar-Laser, Laser-Physics, Reliant 50S-489, 50-W power, wavelength 488 nm) in an uncoated fused-silica capillary (28.5/20.0 cm, 50-μm i.d.; Composite Metal Services, Ltd., U.K.). The capillary was positioned in still air without thermostating. The detector was placed at the cathodic side of the capillary. The emitted light was focused by a microscopic lens system and passed through a filter (cutoff 500 nm). Light intensity was measured with a photomultiplier (Hamamatsu H5785, Shimokanzo, Japan). Injection was carried out hydrodynamically by lifting the capillary inlet by 3 cm for 10 s. Separation was at +7.5 kV at ambient temperature. Data collection was done with DataApex software (Prague, Czech Republic). The capillary was conditioned by flushing between all runs with ~20 μL of background electrolyte (BGE).

Capillary electrophoresis with UV absorbance detection was performed using an automated HP3D capillary electrophoresis system (Agilent, Waldbronn, Germany) equipped with a diode array detector. A fused-silica capillary (Composite Metal Services, Ltd., U.K.) with a 59.0-cm total length, effective length 50.5 cm, 75-μm i.d. was used. The capillary was packed in a standard HP cassette and thermostated to 25.0 °C during all experiments. Injection was at 200 mbar·s; voltage was +15 kV. Data aquisition, storage, and analysis were performed using Agilent ChemStation Plus software.

Reagents. HRV2 and HRV14 were produced and purified from infected cell pellets, as described previously.[20] Virus concentration in this stock preparation was determined by CE from the virus peak and corrected as described.[21] The viral protein concentration was ~1.4 mg/mL for preparation P1 and 0.2 mg/mL for preparation P2 (buffer was 50 mmol/L Tris). Viral RNA was prepared in vitro by transcription of a full-length cDNA clone of HRV1A following the protocol used for HRV2.[22] Very low density lipoprotein receptor fragment encompassing ligand binding repeat 1−3 fused to maltose binding protein at its N-terminus and to a hexa−his tag at its C-terminus (MBP−VLDLR$_{1-3}$) was produced and purified as described.[23] RiboGreen in DMSO was obtained from Molecular Probes (Eugene, OR) as part of a kit for the determination of RNA concentrations. Bovine pancreatic RNase type 1-AS was from Sigma (Milwaukee, WI). Recombinant RNase inhibitor (RNasin) was from Promega (Madison, WI). All other chemicals were purchased from E. Merck (Darmstadt, Germany) and were used without further purification.

The following buffers were used: (i) Virus separation was carried out in 100 mmol/L boric acid, 10 mmol/L SDS, adjusted to pH 8.3 with NaOH. (ii) RNA separation was made in virus separation buffer without SDS. (iii) Incubation for dye penetration was performed in 50 mmol/L borate buffer, pH 8.3. For CE with UV detection, the samples were diluted 1:10; for CE with LIF detection, 1:1, respectively; in both cases, with virus separation buffer. All solutions were prepared in double-distilled water. Solutions were degassed by ultrasonication for 5 min and centrifuged for 5 min in a tabletop centrifuge (Eppendorf, model 5415D, Hamburg, Germany) at 11800*g* prior to CE analysis.

Procedures. For labeling the RNA within the intact viral capsid with RiboGreen, purified virus was diluted 1:20 with incubation buffer. A 20-μL portion of this solution was incubated with 1 μL of RiboGreen (in DMSO) in the dark at 25 °C or 37 °C for different times. The sample was diluted 1:1 with virus separation buffer containing 2 nmol/L fluorescein as internal standard and analyzed by CE-LIF. To release the genomic viral RNA from the capsid, 20 μL of virus solution was exposed to 56 °C for 1 min by immersion in a water bath.

Digestion of the released RNA was carried out for 45 min after adding 0.5 μL RNAse (concentration 1.5 mg/mL in water) to 10 μL of the heat-treated virus solution. In case that digestion should be inhibited, 1 μL RNAsin (concentration 40 IU/μL) was added to 10 μL of the heat-treated virus solution.

Complexes between HRV2 and the soluble receptor fragment (MBP−VLDLR$_{1-3}$) were formed by reaction of the components at 37 °C for 1 h.[24] The receptor was added at a molar excess of

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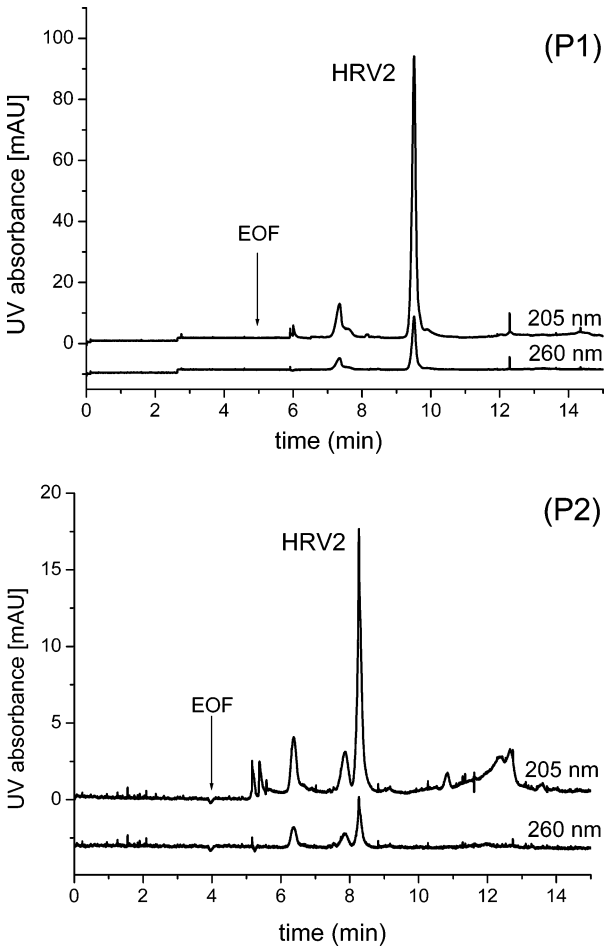

Figure 1. Electropherograms with UV detection at 205 and 260 nm, respectively, of two HRV2 preparations of different purity. Separation conditions: fused-silica capillary, 59.0-cm total length, effective length 51.5 cm, 75-μm i.d. Temperature, 25.0 °C; separation buffer, borate 100 mmol/L, pH 8.3, 10 mmol/L SDS. Injection at 200 mbar·s; separation voltage, 15 kV.

200, 400, and 1600 over the virus. The mixture was then diluted 1:1 with separation buffer, an aliquot was injected into the capillary, and CE-LIF was performed in virus separation buffer. The entire procedure was essentially the same as described in previous papers.[24−27]

RESULTS AND DISCUSSION

Time Course of RiboGreen Penetration into the Viral Capsid. To bind the viral genomic RNA, the dye has to diffuse through the proteinaceous shell into the core of the virus. There is no reason to assume that this is an active process; thus, it is most probably diffusion-controlled, although it is not known whether the nature of a given compound (hydrophobic, hydrophilic, ionic, etc.) has any influence. However, although the pores presumably opening at the 5-fold axes of icosahedral symmetry

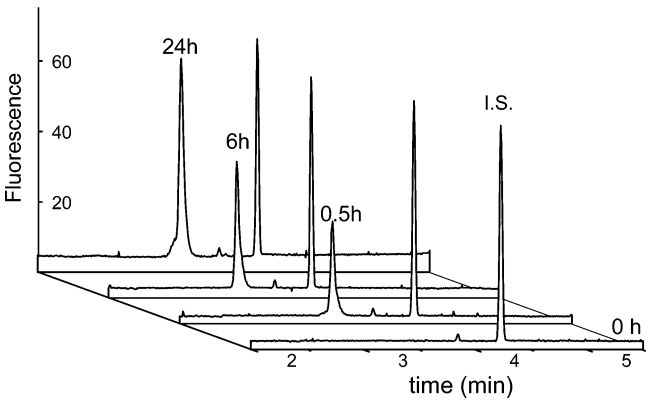

Figure 2. Time dependence of fluorescence labeling of HRV2-RNA with RiboGreen. Native HRV2 (preparation P2) was incubated with the dye at 37 °C, and aliquots were withdrawn at the times indicated and analyzed by CZE with LIF detection (ex/em: 488/520 nm). I.S., internal standard, fluorescein at 1 nmol/L. Experimental conditions: uncoated fused-silica capillary (28.5/20.0 cm, 50-μm i.d.), positioned in still air without thermostating. Separation buffer was as in Figure 1. Hydrodynamic injection by lifting the injection inlet by 3 cm for 10 s. Separation voltage, 7.5 kV. The peak of RiboGreen eluted after 5.5 min.

upon RNA release[28] have been estimated by molecular dynamics methods to be up to ∼2 nm in diameter,[29] nothing is known about parameters underlying mass transport from the surrounding solution through the proteinaceous shell, especially if the virus is in its native conformation. Since we can assume that both diffusion and "breathing" depend on the temperature, the efficiency of complex formation between the dye and RNA inside the intact virus is expected to increase with temperature. To not induce an irreversible structural modification of the virus (HRV2 is converted to empty "B" particles at >50 °C[11,13,30]), the experiments were conducted at room temperature (25 °C) and at 37 °C.

Two HRV2 preparations were used for the following experiments. As can be seen from their electropherograms recorded at 205 and 260 nm (Figure 1), they differ in purity. Preparation P1 shows one main peak characteristic for the virus (as confirmed by many means; see our previous papers) and only one major impurity (the peak at 7.3 min migration time). The more contaminated preparation P2, on the other hand, shows several impurities; the highest peak stems from the virus. In addition, the virus concentration was ∼7 times lower, as compared to the preparation P1 (note the different scales of the *y* axes).

Both preparations were incubated with the dye for different time periods at 37 °C and analyzed by CE-LIF. At time $t = 0$ (analysis immediately after mixing the components), no fluorescing peak at the position corresponding to the electrophoretic mobility of the virus [21] was seen (Figure 2, 0 h). A clearly discernible peak appeared after 0.5 h of incubation and increased with longer incubation time. When the same experiment was carried out at 25 °C, only a very small peak was seen, even after 24 h of incubation (data not shown). Because the dye specifically

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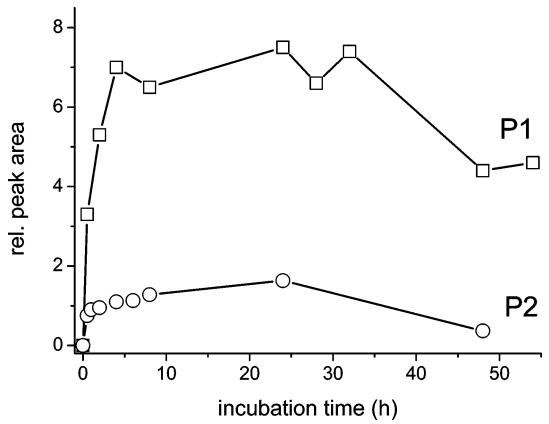

Figure 3. Time-dependent change of the fluorescence signal of HRV2 (preparations P1 and P2) upon incubation with RiboGreen at 37 °C. At the times indicated, aliquots were analyzed by CE-LIF. The relative peak area is the ratio of virus peak area to the peak area of fluorescein added as internal standard (1 nmol/L). Experimental conditions as in Figure 2.

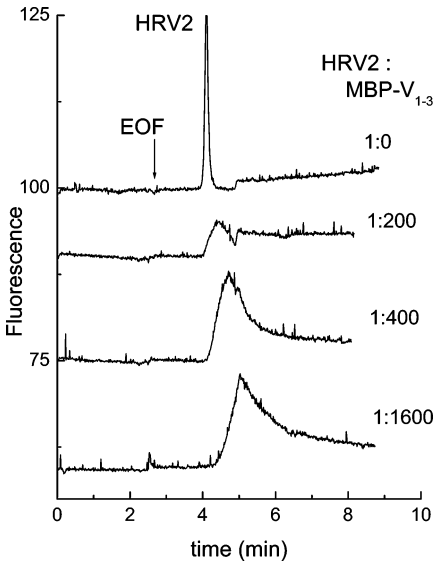

Figure 4. CZE with LIF detection of HRV2 incubated with RiboGreen followed by reaction with the soluble receptor fragment MBP−V_{1-3} that specifically binds to native 150S virus particles. The reaction was carried out at a constant virus concentration (1.6 nmol/L HRV2 preparation P1) with the receptor at an approximate molar excess, as indicated. Separation conditions as in Figure 2.

stains polynucleotides and the electrophoretic mobility of the fluorescent material is identical to that of the virus ($\sim 23 \times 10^{-9}$ $m^2\ V^{-1}\ s^{-1}$), it is plausible that the signal stems from labeled viral RNA within the HRV2 capsid.

To determine the time course of fluorescence increase at 37 °C in more detail, the two virus preparations were again incubated with RiboGreen, and samples were taken at the times indicated in Figure 3 and analyzed as in Figure 2. For both preparations, the relative peak area of HRV2, as related to that of the internal standard, increased strongly within the first 2 h of incubation then plateaued to slightly decreased upon extending the incubation time over 24 h. This decrease is most probably due to denaturation, degradation, or aggregation of the material.

Material in the CE Peak Binds Viral Receptor. To confirm that the peak observed by CE-LIF corresponds to native virus (see Figure 2), we made use of its specific binding to the VLDL receptor. VLDLR, among other members of the LDLR family, is used by HRV2 to gain access to the cell. Soluble recombinant fragments of this protein were shown to form stoichiometric complexes with HRV2,[20] which are detectable by CE, since their mobility differs from that of the virus.[24] It is important that subviral (135S and 80S) particles do not bind. This allows discrimination between A particles (which also contain RNA but lack VP4) and native virus.

Samples of virus preincubated with RiboGreen were thus allowed to react for 1 h with MBP−$VLDL_{1-3}$ at different molar ratios at 37 °C prior to CE analysis, as described in our previous paper.[24] Aliquots of the reaction mixtures were then analyzed by CE. The electropherograms obtained are shown in Figure 4. The shift of the peak as a function of receptor amount added clearly indicates that a complex was formed; its broadening most probably results from heterogeneity (a mixture of complexes with dissimilar ratios between virus and receptor). A similar behavior was noted previously.[24] It can not be excluded that the complex partially dissociates during the CE run. Nevertheless, the shift of the fluorescent peak upon addition of recombinant receptor strongly suggests that the fluorescent material indeed represents HRV2.

The Virus Peak Disappears upon Heating at 56 °C. HRVs are heat-labile; at temperatures exceeding 50 °C, rhinoviruses quickly release their RNA and give rise to subviral B particles.[11] Heating of the virus should thus lead to extrusion of the fluorescence-labeled RNA with a concomitant disappearance of the peak tentatively assigned to the virus. Note that the remaining empty capsids exhibit only native fluorescence originating from tryptophan and tyrosine present in the viral proteins; these have excitation wavelengths around 280 nm and emit at 360 and 310 nm, respectively, wavelengths which are all outside the working range of the LIF detector. First experiments showed that exposure of the sample to 56 °C indeed resulted in loss of the peak. However, no new peak appeared. There are several explanations for this finding: (i) The peak is rather the result of an artifact than the complex formed between the encapsulated RNA and RiboGreen. (ii) The RNA−RiboGreen complex is formed, but the buffer system is not suited to the analysis of the free RNA−RiboGreen complex. (iii) Although the RNA−RiboGreen complex is formed inside the virus capsid, it quickly dissociates after its release from the capsid. (iv) The released RNA is rapidly degraded by contaminating RNases present in the buffer (note that RNases cannot digest RNA inside the capsid). We thus tested whether free RNA could be detected under the separation conditions used.

BGE Suited for the Analysis of an RNA-RiboGreen Complex. First attempts to analyze RiboGreen−RNA complexes from synthetic viral RNA in virus separation buffer failed, and only the RiboGreen peak was detected (it was found that the presence of 10 mmol/L SDS in the separation buffer increased the intrinsic fluorescence of RiboGreen by a factor of 7). To prevent digestion in the BGE, which was not made RNase-free, we added RNasin to the BGE. Since RiboGreen is hydrophobic, we reasoned that the SDS present in the buffer used for virus analysis might dissociate it from the RNA. Therefore, we omitted the detergent in the following experiments. Indeed, when in vitro transcribed viral RNA was incubated with RiboGreen and analyzed in borate buffer without SDS but containing the RNase inhibitor RNasin, a

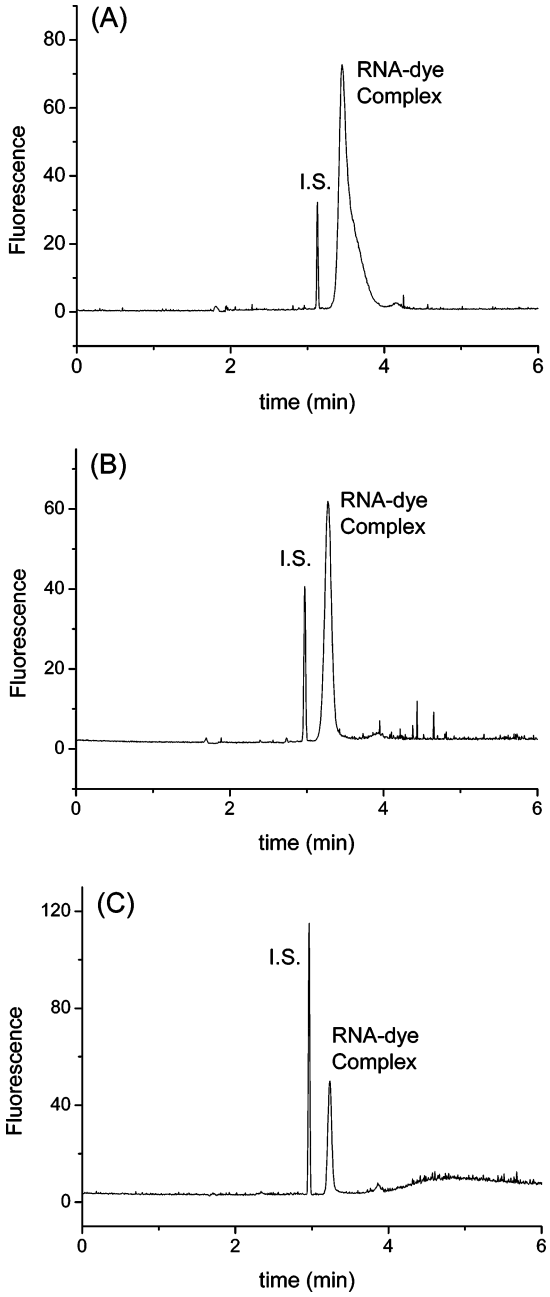

Figure 5. CZE with LIF detection of the complex between RiboGreen and viral RNA: (A) Complex with free synthetic viral RNA. (B) HRV2 (preparation P1) was incubated with RiboGreen for 20 h at 37 °C, and the RiboGreen−RNA complex was subsequently released from the virus by exposure to 56 °C for 1 min in borate buffer (100 mmol/L, pH 8.3; no SDS) supplemented with RNasin. (C) HRV2 (preparation P1) without RiboGreen penetration was heated to 56 °C for 1 min in borate buffer in the presence of RNasin, and the eventually released RNA was reacted with the dye prior to analysis. I.S., internal standard, fluorescein at 1 nmol/L.

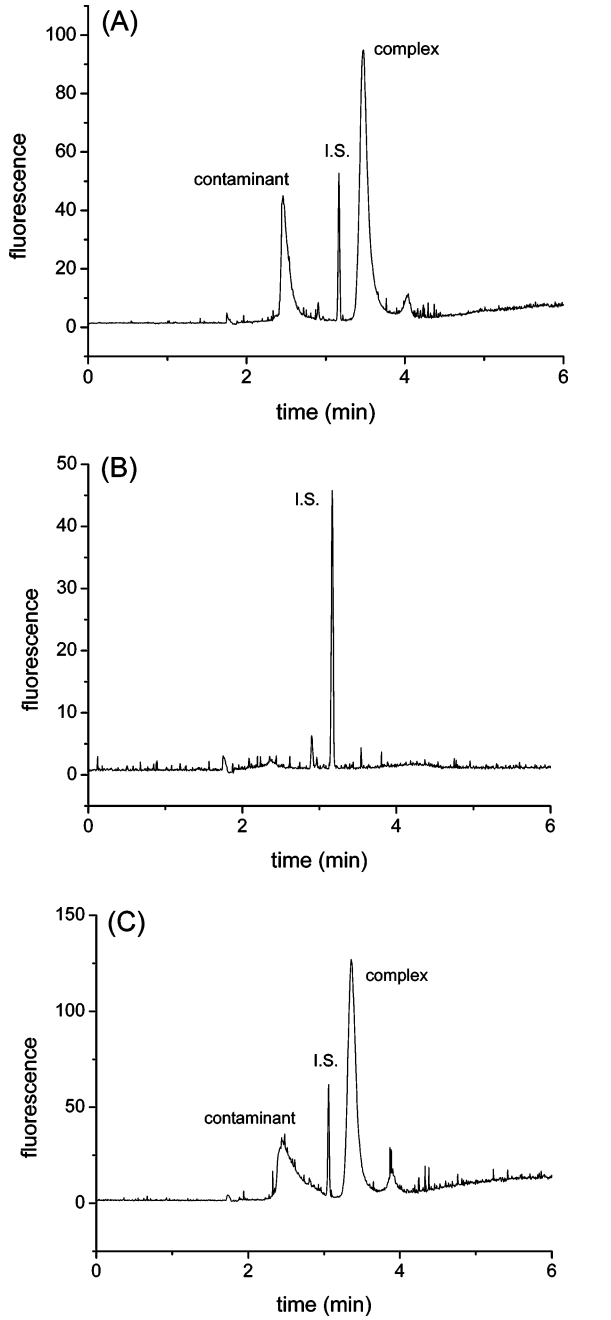

Figure 6. RiboGreen complexed RNA disappears upon digestion with RNase. HRV2 (preparation P2) was incubated with RiboGreen for 20 h at 37 °C and then heated to 56 °C for 1 min to release the labeled RNA. Samples were analyzed by CZE with LIF detection in RNA separation buffer (that does not contain SDS). (A) The peak of the complex is indicated. (B) As in (A), but the sample was subjected to digestion with RNase for 45 min at 37 °C prior to analysis. (C) The sample was treated as in (B), but in the presence of an excess of RNasin (see Figure 5B for comparison). Note that the virus sample P2 used was less pure than preparation P1 and contained an unidentified contaminant, presumably RNA, since the corresponding peak disappeared upon incubation with RNase in the absence (B) but not in the presence (C) of RNasin. I.S., internal standard, fluorescein at 1 nmol/L.

fluorescent peak appeared with mobility expected for free RNA (Figure 5A). Unfortunately, buffer lacking SDS is not suitable for CZE of the virus; the detergent is required to avoid aggregation and wall adsorption of HRV2.[21] Therefore, we abandoned detection of the virus at the benefit of being able to detect the RNA.

Release of Labeled RNA upon Heat Denaturation of the Virus. Virus was incubated with RiboGreen at 37 °C for 20 h to allow for penetration; the mixture was then heated to 56 °C for 1 min to release the genomic RNA, and the sample was analyzed by CE-LIF. As seen in Figure 5B, this treatment led to the appearance of a peak (using the RNA separation buffer) with a migration time identical to that of the complex between synthetic

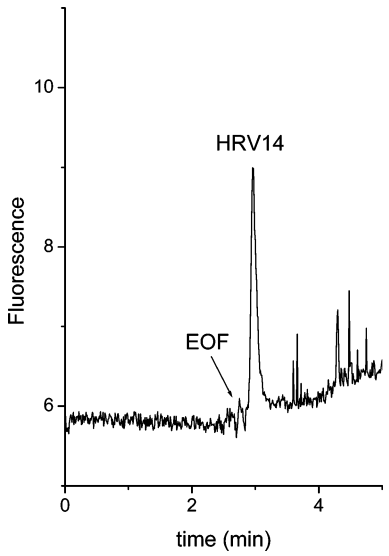

Figure 7. RiboGreen penetration of HRV14 to be compared to that of HRV2 (see Figure 2). Experimental conditions were as in Figure 2.

RNA and RiboGreen (Figure 5A). This strongly suggests that the RNA had become labeled when still within the viral capsid (compare to Figure 2) and was released as such upon heating (Figure 5B).

For comparison, we heated native virus without prior RiboGreen penetration to 56 °C for 1 min to release the (unstained) viral RNA into the incubation buffer. Because this solution contains RiboGreen, the complex between RNA and dye should also form directly after the release of the RNA from the virus, which is, indeed, observed (Figure 5C).

To further confirm the peak as the RNA−dye complex, the sample was incubated with RNase. HRV2 preparation P2 was incubated with RiboGreen, and the RNA−dye complex was released as described above by heating to 56 °C for 1 min (Figure 6A; note the contaminant peak). When RNase was added (Figure 6B) to the same sample, the RNA peak seen in Figure 6A disappeared, but it remained unchanged when RNasin was added prior to addition of RNase (Figure 6C). As indicated, an additional large fluorescent peak was present that also disappeared upon RNase treatment. This is presumably an RNA contaminant present at comparably high concentration in virus preparation P2.

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Penetration of Another Viral Serotype. To assess whether RiboGreen penetration does also occur in other viral serotypes, the same procedure was applied to HRV14, which belongs to the rhinovirus major receptor group. Incubation of HRV14 with RiboGreen resulted in a peak closely after the EOF with an electrophoretic mobility identical to that of untreated HRV14, $\sim 10 \times 10^{-9}$ m^2 V^{-1} s^{-1}.[26] This demonstrates that RiboGreen labeling of viral RNA within the capsid is applicable to different HRV serotypes. It might even be used for an easy identification of different viral strains.

CONCLUSIONS

In accordance with results of other groups,[16,19,31,32] it is shown here by CE that a small-molecular-mass compound can penetrate into the capsid of two different rhinovirus serotypes in a time- and temperature-dependent manner and bind to the genomic RNA. No irreversible structural changes of the viral capsid were seen in this procedure, as demonstrated by the binding of receptor fragments that only attach to native virions. When the virus was exposed to 56 °C for 1 min, the RiboGreen-labeled RNA was released, as evidenced by the disappearance of the corresponding peak upon digestion with RNase. The peak remained, however, in the presence of RNasin, an inhibitor of RNases.

In accordance with the information given by the supplier, RiboGreen has a very low intrinsic fluorescence. However, we observed that it increased substantially in the presence of SDS (data not shown); this dye peak did not interfere with the analysis of virus or RNA, because it has a largely different electrophoretic mobility, $\sim 42 \times 10^{-9}$ m^2 V^{-1} s^{-1}, eluting at longer migration time.

In summary, a method is presented that allows labeling the genomic RNA of native rhinoviruses without inducing irreversible structural changes or modifications at the viral surface. It is promising as a tool for the production of fluorescent virus for cell-binding and internalization studies on the single molecule level.

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