Ran GTPase has been shown to be involved in host innate immune response, and two alleles, RanT/n and RanC/d, which differ from each other by a single nucleotide, have opposite effects on host innate immune response. In this study, we showed that although intravenous administration in mice with either Ran cDNA using an identical adenovirus (Ad) vector resulted in no significant difference in vector tissue distribution, intraperitoneal administration resulted in effective vector transduction into peritoneal macrophages, coupled with a striking difference in vector tissue distribution in 2 h or less. We further demonstrated the presence of prepackaged RNA in virions of Ad vectors, in cells actively producing Ad virus particles, and in cells very shortly after Ad infection. Real-time PCR analysis confirmed the presence of prepackaged RNA and estimated the copy number to be one per viral genome. The prepackaged viral mRNA could be used for translation into proteins, as shown by experiments in which the transcriptional inhibitor actinomycin-D was used. Hence, translation of Ran proteins from prepackaged viral mRNA immediately after virus uncoating in the cytoplasm is one mechanism that would account for an early difference in Ad-vector tissue distribution after efficient gene transfer into macrophages.

Adenoviruses (Ad) are non-enveloped, double-stranded DNA viruses with a linear genome of 30–40 kb. There are about 50 serotypes of Adenoviridae, and Ad2 and Ad5 within group C viruses are the most common types used in gene therapy applications (1, 2). In this context, recombinant replication-deficient Ad vectors have distinct advantages including the ability to package large therapeutic genes of interest, ease of handling, high titer production, and broad cell tropism. The major disadvantage has been the ability of Ad vectors to trigger immune toxicities characterized by both innate and adaptive immunity in the host (1, 2, 4, 5).

Innate immunity involves neutrophils and professional antigen-presenting cells (APCs), such as tissue macrophages and dendritic cells. When activated, they produce a number of proinflammatory cytokines, mainly tumor necrosis factor-α, interleukin-1, and interleukin-6, which then lead to the production of a whole host of soluble factors, including complement, antimicrobial agents, and other cytokines. Professional APCs have an active phagocytic activity and are efficient in capturing foreign microbes and processing antigens. This is triggered by their initial interaction with pathogens (in this case, Ad antigens) via pathogen-associated molecular patterns. After activation of phagocytosis and antigen processing, the antigen-loaded APCs begin to express major histocompatibility complex class II molecules and will then migrate to draining lymph nodes, directed by chemokines. Adaptive immunity involves activation of T cells, which requires two signals (4). The first signal begins when antigen-loaded APCs encounter T cells via major histocompatibility complex class II molecules. This results in further enhanced interactions between T cells and the activated APCs, via co-stimulatory molecules, which in turn lead to the delivery of a second signal. Activation of cell-mediated immunity via these T cells results in the elimination of Ad virus and virus-transduced cells in the host.

Various improvements have been made regarding engineering of Ad vectors for gene therapy. These efforts successfully minimized host adaptive immune response to the vectors and have improved the efficacy of gene transfer and duration of therapeutic gene expression. Host innate immune response to Ad vectors, however, remains a significant concern. The difficulty lies upon the fact that host innate immune response is induced shortly after adsorption and entry of the recombinant Ad virus; the response is therefore directed toward viral capsid proteins and is independent of Ad vector engineering technology.

Adsorption of Ad is rapid and can occur in less than 1 min (6). This is achieved via binding of Ad fiber knob protein to a high-affinity Coxsackie virus–adenovirus receptor on target cells. After this high-affinity binding, viral internalization begins with a low-affinity interaction between an arginine-glycine-aspartic acid (RGD) motif on the Ad penton base capsid protein and αv integrins on APCs, followed by receptor-mediated endocytosis (1, 7, 8). Ten minutes after internalization, pH-dependent acidification of endosomes occurs, allowing Ad virions to enter the cytoplasm and to uncoat (9). By 30–40 min, uncoated virions reach the nuclear pore complex, and transcriptional activation of viral genes is known to take place about 2 h after viral adsorption (1, 10, 11).

Despite Ad viral transgene expression, thought to begin 2 h after virus internalization (described above), infected macrophages become activated and produce mRNAs of proinflammatory cytokines as early as 30 min after infection (6). This rapid innate immune response from infected host cells has been commonly observed (5, 12–16). It is a major barrier in adenoviral gene therapy even though important technological advances in vectors development have been successful in eliminating toxicity caused by adaptive immunity directed against Ad vectors or vector-transduced cells.

We have previously shown that Ran GTPase is involved in host innate immune responses. Adenoviral transfer and ex-
preparation of RanC/d allele leads to down-modulation of proinflammatory cytokine production in vitro and in vivo and protection against septic shock in mice (17–20). In this report, we present data to demonstrate a difference in tissue distribution between RanC/d- and RanT/n-transduced cells in 2 h or less after infection. These data suggest that a difference in biological functions is already present very early on after viral infection, even on the two vector-transduced cells, as our previous publication suggested. We further showed that prepackaged mRNAs are present in virions of Ad vectors, in Ad-virus producing cells, and in cells immediately transduced with Ad vectors. Release of prepackaged Ad mRNAs into cytoplasm, followed by translation of mRNA of viral transgenes after endosome disruption, would explain the rapid biological functions of Ad transgene even without transcriptional activation of viral transgenes.

MATERIALS AND METHODS

Animals and Adenovirus Vectors—We purchased CD-1 outbred mice from Charles River Laboratories, and C3H/HeOuJ, C3H/HeJ, and Balb/c inbred mice from Jackson Laboratories. Typically, we used mice that were 5–8 weeks old after a 2- to 3-week acclimatization.

All adenovirus stocks were prepared from human embryonic kidney 293 cells as described previously (17, 18). The stocks were purified by CsCl banding twice, dialyzed, aliquoted, and stored at −80 °C until use. Mice were inoculated either intraperitoneally or intravenously with 5 × 10^10 plaque-forming units/mouse of either Ad-Ran C/d or Ad-Ran T/n virus. At 2 h after infection, the animals were sacrificed; peritoneal cells, livers, lungs, hearts, spleens, peripheral blood, and kidneys were harvested from each animal. Tissue DNA was extracted using DNAzol reagents (Invitrogen) and PCR analysis was conducted, using primers homologous to Ran (see PCR section below).

PCR or RT-PCR—PCR or reverse transcription (RT)-PCR was used for the detection of viral vectors or their messages, respectively, in both animal tissues and cell lines. The following is a summary of the reaction conditions used: for adenovirus carrying the green fluorescent protein (Ad-GFP), the PCR was conducted using primers homologous to regions on the enhanced green fluorescent protein gene that would result in a 265-bp product. The sequence for the sense primer, EGFP1, is 5′-ACGACGGCACTACAGACCCG-3′ and the antisense primer, EGFP2, is 5′-TCTATATCATGGCGAGAAGC-3′. The PCR reaction conditions for 30 cycles were as follows: denaturation at 95 °C for 1 min; annealing at 60 °C for 30 s; and extension at 72 °C for 30 s. For Ad-Ran C/d or Ad-Ran T/n, the PCR was conducted using primers homologous to regions on the 1.1-kb Ran C/d or Ran T/n insert that includes a 39-bp HA tagging sequence resulting in a 158-bp viral band distinguishable from the 119-bp endogenous Ran band. The sequence for the sense primer was 5′-TTGTGTCGATGCCTCTTGTG-3′ and for the antisense primer was 5′-GGCTCATCAGTCTCAGTGGGA-3′. The PCR reaction was carried out as above. The PCR products were then analyzed on a 2% agarose gel, yielding two products: the first, a 119-bp band, represented the endogenous Ran; the second product, a 158-bp band, represented the exogenous or viral Ran.

For RT-PCR, approximately 2.5 × 10^12 Ad-GFP virus particles were lysed in 500 µl of buffer containing 0.001 M EDTA, 0.01 M Tris-HCl, pH 7.5, and 0.1% SDS at 57 °C for 10 min with shaking (21). Phenol/ chloroform extraction, pH 4.5, was conducted on the particle lysate followed by ethanol precipitation. The sample was then resuspended in 200 µl of diethyl pyrocarbonate-H_2O. Two 50 µl aliquots were removed and digested with DNase (Roche) at a concentration of 4 units/µl at 37 °C for 90 min in a final volume of 40 µl. Reverse transcription was then conducted on 20 µl of the sample, as described by the manufacturer, using SuperScript II reverse transcription kit (Invitrogen). PCR was conducted as described above, and two sets of the PCR products were analyzed on a 2% agarose gel.

In Vivo Infection of Human 5637 Cells—Human embryonic kidney 293 cells were allowed to grow to 80% confluence in a 175-cm² flask (Corning) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The flask were then infected with 2 ml of diluted Ad-GFP virus (in Dulbecco’s modified Eagle’s medium with 2% fetal calf serum) at an m.o.i. of 200 for 1 h at 37 °C with gentle shaking. Dulbecco’s modified Eagle’s medium (10% fetal calf serum) was then added to a final volume of 10 ml, and the flask were allowed to incubate at 37 °C. At 6, 12, 18, and 24 h after infection, 0.5 µl/µl PurP (Amersham Biosciences) was added to the 175-cm² flasks at a concentration of 20 µg/ml and allowed to incubate for 6 h at 37 °C. After incubation, the cells were harvested, washed with phosphate-buffered saline, and lysed by three rounds of freezing/thawing cycles. Next, the cell lysate was centrifuged at 12,000 × g for 10 min to remove cell debris and then purified by binding the virus 2× in a CsCl gradient (0.5 g/ml). The purified virus was then desalted using a Centricom filter column (Millipore) and then treated with RNase (5 units/ml) (Invitrogen) at 37 °C for 30 min. A 10-µl fraction of the particle suspension was then trichloroacetic acid-precipitated and then redissolved in water.

Actinomycin D Treatment—293 cells were seeded 2 days before infection in 100-mm tissue culture dishes. On the day of infection, one dish was trypsinized for cell counts. Cells were mock-infected or infected with Ad-LacZ virus at an m.o.i. of 10^3 in the presence or absence of 5 µg/ml of actinomycin D (Sigma). The amount of actinomycin D used was shown to effectively suppress cellular transcription under similar infection protocol (22). Infection was carried out in 3 ml of Dulbecco’s modified Eagle’s medium supplemented with 2% fetal bovine serum per 100-mm dish for 3 or 6 h with continuous shaking to achieve most efficient infection (21). At the end of infection, cells were washed two times with cold phosphate-buffered saline and scraped off the dish and lysed immediately in a small volume of reporter lysis buffer (β-galactosidase enzyme assay system; Promega) and assayed for β-galactosidase activity following the manufacturer’s protocol. Standard curve was generated using included β-galactosidase standard, and activity in unknown samples was analyzed using Prism software (GraphPad Software). One unit is defined as the amount of β-galactosidase activity able to hydrolyze 1 µmol o-nitrophenyl-β-D-galactopyranoside to o-nitrophenol and galactose in 1 min at pH 7.5 at 37 °C.

Quantitation of Prepackaged Viral RNA by Real-time PCR—Total RNA was extracted from 2.5 × 10^12 plaque-forming units (Ad-RanTn)/H_2O. Lysate was collected after lysis of virions in Tris-EDTA buffer (0.001 M EDTA and 0.1 M Tris-HCl, pH 7.5) containing 1% SDS, at 55 °C for 10 min with shaking, phenol/ chloroform, pH 4.5, was extracted twice, and EtOH was precipitated. The RNA pellet was dissolved in 200 µl of RNase-free water. To eliminate genomic or viral DNA contamination, half of the RNA sample was treated with 500 units of RNase-free DNase (Roche) for 90 min at 37 °C to determine the extent of DNA contamination in each RNA sample, whereas the other half was left untreated. To prepare cDNA, 10 µl from each RNA sample were used in RT reaction with Ran-specific reverse primer (5A) in a final volume of 20 µl. After 10-fold dilutions of each cDNA product, 10-µl RT products were used for real-time PCR, using an Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primers and TaqMan probes (Fig. 5A) were designed with Primer Expression Software and Assay-by-Design (Applied Biosystems) and purchased from Invitrogen and Applied Biosystems, respectively. Real-time PCR was conducted with triplicates, each in a 25-µl TaqMan Universal PCR master Mix (Applied Biosystems), together with 200 nM primers and 200 nM probe. After denaturing at 94 °C for 5 min, the reaction was run for 40
cycles with denaturation at 94 °C for 20 s, annealing at 55 °C for 20 s, and primer extension at 72 °C for 30 s, except at the last cycle, where final extension was set at 72 °C for 10 min. As a negative control, another RNA sample was directly and simultaneously subjected for PCR reaction. Serial 10-fold dilutions of known amount of plasmid DNA (pCD-RanT/n-HA) were used as positive control to generate the standard curve.

**RESULTS**

**Tissue Distribution of Viral DNA Affected by the Route of Inoculation**—We showed previously that expression of RanC/d in macrophages in vitro resulted in reduced production of tumor necrosis factor-α and that intraperitoneal inoculation of our Ad vector carrying RanC/d protected mice from septic shock (17, 20, 23). Because the peritoneal cavity contains mainly resident macrophages, we sought to determine whether intraperitoneal administration would lead to effective down-modulation of host innate immune response. We therefore compared the result of different routes of inoculation. For each mouse, we inoculated either Ad-RanT/n or Ad-RanC/d at a dose of 5 × 10^10 plaque-forming units, administered intravenously or intraperitoneally. We analyzed DNA extracted from various organs of each mouse using a competitive quantitative PCR analysis in which the intensity of the amplified endogenous Ran DNA in each sample was compared with that of the amplified viral Ran DNA in the same sample (18). By this design, equal intensity of viral Ran DNA in the same sample (18). By this design, equal intensity of viral Ran DNA compared with endogenous Ran DNA in a particular sample would suggest that the copy number of Ad viral DNA copy number is more than one copy per haploid genome.

For intravenous administration, both Ad vectors resulted in similar amounts of amplifiable viral Ran DNA from multiple organ samples (i.e. bone marrows, livers, peripheral blood, and spleens), with highest intensity in livers and spleens (not shown). Whereas the signals in livers and spleens were consistently intense, those in peritoneal macrophages were poor. These data are similar to observations from human clinical trials in which Ad vectors were administered intravenously and multiple and nonspecific organs were transduced by a number of genetic vectors with similar observations (5, 6, 24, 27). However, intraperitoneal administration of adenovirus vectors yielded very interesting results. A predominant viral band in peritoneal macrophages was obvious for both vectors (Fig. 1). Transduction of peritoneal cells seemed to be highly efficient, because the amplified endogenous Ran fragment was either weak or absent, whereas the amplified viral Ran DNA was very intense. Giemsa-Wright staining of cytoprep smears indicated that 80–90% of the peritoneal cells are monocytes/macrophages. In addition, the intensity of the amplified viral Ran DNA in other organ samples seemed very different between RanT/n and RanC/d. Intense viral bands were observed in livers and spleens of RanT/n-injected mice, whereas a faint band was seen in livers but not spleens of RanC/d-infected mice.

**Fig. 2. Presence of prepackaged RNA in adenoviral virions.** A, RT-PCR. Ad-GFP viral particles in lanes A and B were lysed, treated with 4 units/µl DNase (Promega) predetermined to optimally remove viral DNA. The resultant RNAs were purified and subjected to RT-PCR analysis, using enhanced green fluorescent protein-specific primers that would yield a 265-bp band. A positive control (lane H1) was included where the viral DNA in 5 µl of the original viral lysate before DNase treatment was subjected to direct PCR. A negative control (lane 1) was included in each reaction. B, labeling of viral RNA. EIA-positive human embryonic kidney 293 cells, with mock or Ad-GFP infection, were labeled with [α-32P]UTP (100 Ci/2.0 × 10^5 cells) for a period of 6 hours at different time points after infection (i.e. 6, 12, 24, and 45 h). Viral RNA was extracted and treated as detailed under “Materials and Methods.” Incorporation of [α-32P]UTP into viral RNA was examined by trichloroacetic acid precipitation and subsequent scintillation counting. The graph represents the average of three identical experiments, shown with S.D. of the mean.

C, viral RNA inside infectious virions. Non-adenovirus-producing human carcinoma 5673 cells were also infected with Ad-GFP, and RT-PCR was performed at various time points after infection. The GFP band was present only with reverse transcription (+RT) and not without (−RT).
The differences observed between different routes of Ad vector administration and the effective vector transduction of peritoneal macrophages and different tissue distribution of the two vectors via the intraperitoneal route strongly suggest that the differential band-intensity between T/n-mice and C/d-mice after intraperitoneal inoculation is related to differences in biological functions. Such biological differences have been extensively documented in our recent studies (17–20).
Presence of Viral mRNA of the Transgene Prepackaged in Adenovirus Virions—The early and rapid biological changes between Ad-RanT/n and Ad-RanC/d administration prompted us to investigate the nature of adenoviral transgene expression. The time from adenoviral infection to expression of viral gene is believed to be at least 2 hours after viral adsorption and infection (28). This view could not explain completely the obvious difference we observed in the presence of vector tissue distribution between T/n- and C/d-mice. Alternative mechanisms might exist, and we contemplated the possibility that prepackaged adenoviral mRNA of RanT/n- and RanC/d-mice may be present in virions of recombinant adenovirus vectors, so that soon after viral gene transduction and uncoating, the pre-existing messenger RNA allows for immediate translation into Ran proteins. To investigate this possibility, we took five different approaches to address this issue.

First, we purified the Ad-GFP virus particles. After lysis of the purified virions and DNase treatment of the extracted RNA samples, we performed RT-PCR using GFP-specific primers. A 265-bp band was visible only after reverse transcription (+RT) but not without (−RT), which was predicted if viral GFP mRNA were present in the virions (Fig. 2A). The second approach we took was to metabolically label adenovirus-producing cells, the human embryonic kidney 293 cells, with $^{[32P]}$UTP at various time points after infection with Ad-GFP. Virions from these virus-producing 293 cells were purified by CsCl banding (twice), RNase-(Roche) treated to remove any cellular RNA associated with virions, and lysed; the amount of radioactivity present in viral lysates was determined using a scintillation counter. The $^{[32P]}$UTP counts were much higher in viral lysates of infected cells compared with those of uninfected cells, especially between the 10- and 20-h time points after virus infection (Fig. 2B). In our third approach, we also sought to determine whether mRNAs of transgenes were present in infectious virions. Human 5637 cells were infected with Ad-GFP. At various time points after infection, we performed RT-PCR on RNA extracted from these cells. Results in Fig. 2C indicate the presence of mRNA of the transgene as early as ≤5 min after infection. Taken together, these data demonstrate the presence of prepackaged mRNA of the transgene in virions of adenovirus particles; these prepackaged viral mRNAs may be used for translation soon after viral entry.

The fourth approach was to quantify prepackaged RNA in virions of recombinant adenovirus vectors by using real-time PCR to detect the exact copy number of mRNA in Ad virions (Fig. 3). Real-time PCR is highly sensitive, and its standard curve provides an excellent linear correlation between CT values and copy numbers. Our results confirm the presence of prepackaged RNA and that the copy number is estimated to be no more than one copy per viral genome. Few copy numbers were also detected from the RNA sample without RT as negative control, most probably because of incomplete DNase treatment. RanT/n RNA copy number corresponds to 10% of the number of virions used for RNA isolation. Given the presence of empty or incomplete Ad viral particles in sample preparation and significant losses in mRNA recovery during extraction, it would be reasonable to interpret that a detection of 10% of the amounts of Ad prepackaged RNA may reflect a true value of one copy of prepackaged RNA per genome in each adenoviral particle.

The last approach we took was to infect 293 cells with an adenovirus vector expressing the bacterial β-galactosidase gene Ad-LacZ in the presence or absence of the transcription inhibitor actinomycin D. We chose to work on Ad-LacZ based on convenience, because there is a commercial bioassay kit for detecting β-galactosidase. Three hours after infection and actinomycin-D treatment, β-galactosidase activity was measured. As expected, no such activity was present in mock-infected cultures (Fig. 4). On the other hand, a significant amount of β-galactosidase activity could be detected in actinomycin-D-treated cultures that were infected with Ad-LacZ, strongly suggesting the presence of pre-existing Ad-LacZ mRNA after infection. Further experiments choosing a time point as early as 30 min after Actinomycin-D treatment yielded similar results (data not shown).

DISCUSSION

Transthrocheal instillation of adenovirus vectors leads to a rapid uptake of virus particles by murine alveolar macrophages, and elevation of mRNA of proinflammatory cytokines could be detected as early as 30 min after inoculation in alveolar macrophages but not airway epithelial cells (6). Hence, uptake of adenovirus particles by appropriate target cells (i.e. macrophages) stimulates a rapid host cell response. This could not explain completely, however, the early difference in tissue distribution we observed in this study, in that Ad-RanT/n and Ad-RanC/d are identical adenovirus particles, with only a single base difference in Ran cDNA between the two viral genomes. This early difference in tissue distribution of the vector must be biological because it was not observed with intravenous vector administration, and intraperitoneal inoculation effectively transduced tissue macrophages (Fig. 1). Furthermore, in work reported elsewhere, we showed that this route of inoculation introduced the RanC/d transgene effectively into the correct target cells (i.e. macrophages), which resulted in reduced organ inflammation, enhanced protection against septic shock, and decreased serum pro-inflammatory cytokines.

The time from adenoviral infection to expression of viral gene is believed to be at least 2 hours after viral adsorption and infection (28). It has been shown that the binding of adenovirus particles is achieved via a two-step process (1, 3, 9). The first is the high affinity association between viral fibers of adenovirus and the Coxackie virus-Adenovirus receptor of the host cells; the second is the binding of viral penton base protein to a co-receptor of the αv integrin family. Absorption of viral particles can occur

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**Fig. 4. β-Galactosidase activity detected in Ad-LacZ-infected cells.** 3.5 h after infection in the presence or absence of actinomycin D. 293 cells were mock-infected or infected with Ad-LacZ virus with/without actinomycin D as described under “Materials and Methods.” β-Galactosidase activity in cell lysates was assayed using a commercially available kit (Promega) and estimated from standard curve generated in each assay. Similar results were obtained when experiments were repeated.

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2 S. W. Chung, J. L. Song, X. Y. Huang, and P. M. C. Wong, submitted for publication.
in less than 1 min (6). Adenovirion disassembly or uncoating is a step-wise process beginning at the cell surface, where the binding of penton base proteins with integrin receptors induces the release of fiber proteins from the Coxsackie virus-Adenovirus receptor. After internalization of virus particles into endosomes via clathrin-coated pits, the drop in pH leads to rupture of endosome membrane and thereby the entry of partially uncoated virions into the cytoplasm (9). By this mechanism, it is unlikely that such early biological differences could be observed between T/n- and C/d-mice. The presence of prepackaged adenoviral mRNA in the cytoplasm of transduced cells therefore presents an additional mechanism that can explain the rapid protein synthesis from mRNA of the viral Ran transgenes and hence their different biological consequence. Our finding of the prepackaged mRNA of adenovirus, which is a DNA virus, also offers an interesting parallel with a very recent report in which another DNA virus (22). Similarly, these prepackaged mRNAs are thought to be responsible for the initial translation into proteins in the absence of newly made viral gene products.

Prepackaged mRNA in virions of Ad-RanT/n and Ad-RanC/d certainly would help explain the rapid biological difference in the same type of cells transduced with the two vectors with identical genome other than a single nucleotide difference. This must mean that the single base difference is key to this rapid change; a mechanism for such a difference has been proposed in our recent publications (18, 19). Indeed, our most recent RNA in situ hybridization results confirm the existence of such mechanism.3

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3 K. K. Operman, S. W. Chung, and P. M. C. Wong, manuscript in preparation.