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High Level of Transgene Expression in Cell Cultures and in the Mouse by Replication-Incompetent Adenoviruses Harboring Modified VAI Genes

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Replication-incompetent adenoviruses are currently used in gene therapy trials. Most of the work designed to increase the expression from these vectors concerns the modification of *cis* sequences of the foreign transcription unit, so as to improve the transcription level or the stability of the mRNA. In this report, we show that an alternative strategy based on the coexpression of modified VAI genes can efficiently increase gene expression both in cell cultures and in animals. The VAI RNA is synthesized mainly during the late phase of the adenovirus cycle and increases the translation of late adenovirus gene products by counteracting the effect of an interferon-induced kinase, the PKR. We have constructed several modified VAI genes in which the central domain was deleted or substituted by exogenous sequences. These modified VAI genes, or the native VAI gene, were cloned into the left part of adenovirus type 5 genomes harboring their own endogenous VAI gene. One of the resulting viruses (Ad-VAr) increased 12.5- to 502-fold the expression level of reporter genes, either expressed as a constitutive cell line from an extrachromosomal DNA or introduced into cells by coinfection with another adenovirus vector. This effect was independent of the promoter, the coding sequence, and the 5′ untranslated mRNA sequence and was obvious in the two non-E1-complementing cell lines tested (HeLa and Vero). Coinfection of Ad-VAr with adenoviruses expressing the luciferase gene from the major late promoter or Rous sarcoma virus (RSV) promoter by the intravenous route in mice increased by more than 33 (MLP)- to 128 (RSV)- and 4,700 (MLP)- to 30,000 (RSV)-fold the expression level of the reporter gene in the lungs and liver, respectively. The intramuscular coinoculation of Ad-VAr and Ad-MLP-gD (a recombinant adenovirus vaccine expressing gD from the pseudorabies herpes virus) led to a 10-fold decrease in the protective dose of Ad-gD in mice. Ad-VAfull, a similar adenovirus in which the native VAI gene was cloned at the left part of the genome, showed no evidence of efficacy in cell culture and in mice. These results suggest that the use of modified VAI genes expressed at the early phase of the cycle can be helpful in the design of potent adenovirus vectors.

The adenovirus genome harbors two genes encoding small RNAs (VAI and VAII) which are transcribed by RNA polymerase III. VA RNAs accumulate in infected cells in the late phase of infection. The VAI RNA is essential in infected cells for the translation of both viral and cellular mRNAs. This property of VAI RNA is thought to be linked to the inhibition of the action of PKR, an enzyme associated with ribosomes in a complex which associates the factors implied in mRNA translation (for reviews, see references 13 and 14). PKR synthesis is induced by interferon, and its activation is brought about by double-stranded (ds) RNA, which is likely to be present in adenovirus-infected cells because viral mRNAs are transcribed left- and rightward. This activation is a function of the length and concentration of dsRNA. It is efficient at low doses of dsRNA (nanograms per milliliter). In contrast, PKR activation is inhibited at high doses (micrograms per milliliter) of dsRNA. Similarly, dsRNAs longer than 50 bp activate the enzyme. Shorter duplexes (or duplexes containing mismatches) do not activate this enzyme and, at high concentrations, inhibit its activation. The activation of PKR leads to its autophosphorylation. The activated PKR phosphorylates the initiation factor of translation (eIF-2). The blockage of PKR activation occurs at the late phase of infection, by the time RNA VAI accumulates, and most probably before the accumulation of high levels of dsRNA. This blockage of PKR activity counteracts the PKR-mediated inhibition of the translation of viral mRNAs.

The VAI gene has been used to increase the level of protein synthesis from plasmid constructs. Svensson and Akusjärvi (22) demonstrated that the enhancement in translation efficiency was not limited to viral mRNA; nevertheless, this effect seemed to be cell dependent and was significant only in 293 cells, not including HeLa and CV-1 cells. Kaufman (11) demonstrated a more pronounced effect on gene expression controlled by the adenovirus major late promoter (MLP) followed by its tripartite untranslated leader sequence. On the other hand, only a marginal effect was recorded with a replication-competent adenovirus expressing its own VAI gene during infection of cells previously transfected with a plasmid carrying a reporter gene.

With the aim of constructing replication-defective adenoviruses expressing RNA genes under the control of the intragenic VAI promoter, we have inserted several deletion or substitution VAI mutants in the left part of the adenovirus type 5 (Ad5) genome. These Ad5 recombinant viruses retained the wild-type VAI gene normally present in their genome. These viruses were initially dedicated to the expression of antisense or ribozyme sequences but showed unexpected properties, as they were able to increase the level of synthesis of coexpressed genes both in cell culture and in mice very efficiently.

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TABLE 1. Viruses used for the experiments

Name	Transgene (promoter)	Endogenous VAI	E1A-E3	E1B	Reference
Ad-MLP-luc	luciferase (MLP)	Yes	No	No	18
Ad-RSV-luc	luciferase (Rous sarcoma virus)	Yes	No	No	Unpublished data
Ad-MLP-gD	PRV gD (MLP)	Yes	No	Yes	4
Ad-VAemp	VAemp (intragenic promoter)	Yes	No	Yes	This paper
Ad-VAie	VAemp (intragenic promoter)	Yes	No	Yes	This paper
Ad-VAr	VAr (intragenic promoter)	Yes	No	Yes	This paper
Ad-VAfull	VAfull (intragenic promoter)	Yes	No	Yes	This paper

MATERIALS AND METHODS

Cells. The 293 cell line, which is an adenovirus-transformed human embryonic cell line expressing the E1 region of adenovirus integrated in its genome (7), was used for adenovirus transfection, amplification, and titration. African green monkey kidney cells (Vero) and human HeLa cells were used in marker gene expression assays. These cells were maintained in Dulbecco minimum essential medium with 10% fetal calf serum. For all cell inoculations, the virus was kept in contact with cells at 37°C in a low volume of medium for 1 h. The cells were then rinsed and incubated at 37°C.

Plasmids. Several plasmids were constructed according to standard procedures. Plasmid pMLP-luc contained the luciferase gene under the control of the Ad2 MLP joined to its tripartite leader sequence and has previously been described (18). Plasmid pCMV-CAT is similar to pMLP-luc but contains the chloramphenicol acetyltransferase (CAT) gene under the control of the promoter of the cytomegalovirus immediate-early gene. Plasmid p205mMT-CAT (a generous gift from M. R. James, IRSC, Villejuif, France) directs expression of the CAT gene under the control of the murine metallothionein promoter and carries the gene encoding resistance to hygromycin. It also harbors the origin of replication of the Epstein-Barr virus and its EBNA1 gene, leading the plasmid to persist as replicative extrachromosomal DNA in transfected cells.

Viruses. The construction and purification of two replication-defective vectors, Ad-MLP-luc and Ad-MLP-gD (formerly referred to as Ad-gp50), which respectively express the luciferase and the pseudorabies virus (PRV) gD genes under the control of the Ad2 MLP, were previously described (4, 18). All these viruses are E1A and E3 defective. Ad-RSV-luc is identical to Ad-MLP-luc except for the promoter sequence of luciferase gene expression (not shown). Ad-MLP-luc and Ad-RSV-luc are similar to vectors most currently used in gene transfer, as they are also E1B defective. Table 1 details the main characteristics of the viruses.

Generation of recombinant adenoviruses expressing modified VAI genes. These recombinant adenoviruses were constructed following transfection of 293 cells by products of the ligation between plasmids harboring modified or native VAI genes (see Results) and the right *Cla*I fragment of Ad-MLP-gD DNA by calcium phosphate precipitation (Cellphect; Pharmacia). Briefly, Ad-MLP-gD possesses a unique *Cla*I site in the E1A gene. Ligation was performed between the products of the ligation of 5 μg of the linearized plasmid and 5 μg of the large *Cla*I fragment of Ad5 DNA previously purified in a 15 to 40% sucrose gradient. After the cells were overlaid with agar and incubated for 7 to 21 days at 37°C, the resulting virus plaques were picked and viral DNA extracted by a standard procedure was restriction analyzed (8). Viruses were cloned twice by plaque isolation under agar. In order to ensure that no Ad-gD remained, a PCR with primers designed to amplify the gD gene was performed on the DNA of isolates. The recombinant viruses were propagated in 293 cells and purified by cesium chloride density centrifugation. The lack of contamination with $E1A^+$ reverting viruses was checked by PCR with primers specific to the E1A gene of DNA from virus stocks and from infected noncomplementing human cells. Table 1 details the main characteristics of the viruses.

Luciferase and CAT assays. CAT and luciferase activities were assayed in cell lysates with commercial kits (from Boehringer and Promega Biotech, respectively), according to the manufacturer's recommendations. Activities were converted to enzyme concentration by using a curve derived from a serially diluted standard of known concentration.

Detection of VA-derived RNA by RT-PCR. RNA was isolated by Chomczynski's method (2) from adenovirus-infected HeLa cells and then incubated with DNase I. Reverse transcriptase PCR (RT-PCR) was performed with (sense) 5′ AGCGGGCACTCTTCCGTGGTCTG 3′ and (antisense) 5′ AAAACATCCG ACCACCAGGGGT 3′ primers (see Fig. 2). First-strand cDNA was synthesized by incubating samples with avian myeloblastosis virus RT (ONCOR APPLI GENE) at 42°C for 90 min. After avian myeloblastosis virus RT inactivation (80°C for 10 min), second-strand DNA synthesis and DNA amplification were obtained with Super *Taq* DNA polymerase (Stehelin S.A.), with 40 cycles (1 min at 93°C, 90 s at 50°C, and 2 min at 72°C) followed by a final extension step at 70°C for 7 min. RT-PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining.

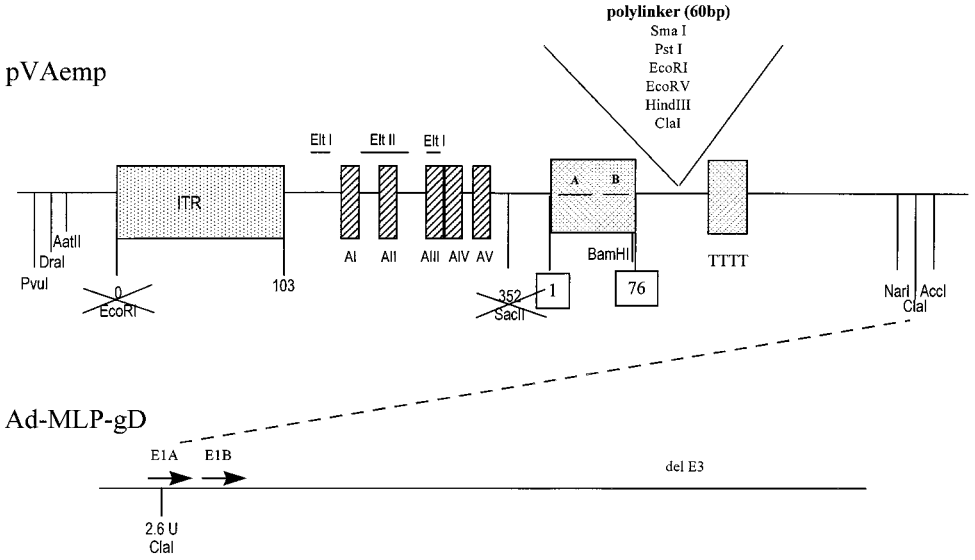

FIG. 1. Structure of pVAemp and Ad-VAemp. See text for the details of the construction of pVAemp. ITR, left inverted terminal repeat of Ad5; Elt I and Elt II, enhancer of E1A; AI to AV, encapsidation signal of Ad5; TTTT, transcription stop signal of RNA polymerase III. Coordinates not in frame correspond to the nucleotide sequence of Ad5. Coordinates in frame correspond to the nucleotide sequence of RNA VAI (nt 1, initiation of transcription). Ad-VAemp was constructed by ligation of pVAemp with the 2.6- to 100-map-unit fragment of Ad-MLP-gD.

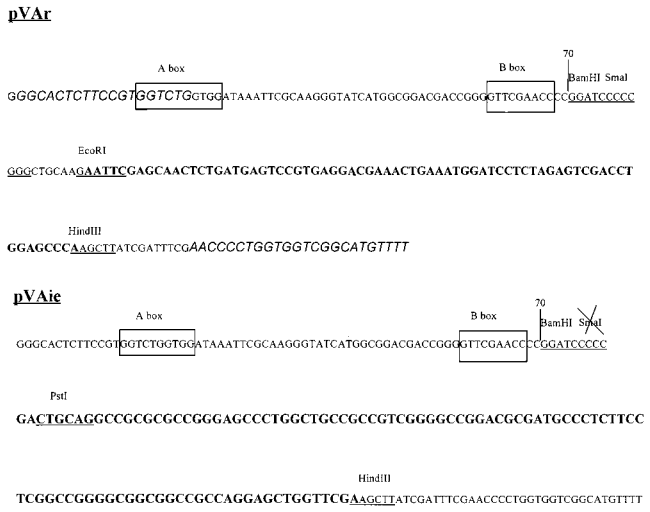

FIG. 2. Nucleotide sequences inserted in pVAemp for the construction of pVAr and pVAie. Boldface indicates sequence inserted in the polylinker of pVAemp; italics indicate nucleotide sequences (or inverse complementary sequences) of the primers designed for detection of VAr by RT-PCR (Fig. 3).

Vaccination experiments. Protocol challenges of mice with the PRV and testing of gD-specific antibodies by enzyme-linked immunosorbent assay have been previously described (3).

RESULTS

Construction of recombinant adenoviruses expressing native or modified VAI genes: Ad-VAemp, Ad-VAr, and Ad-VAie. We started by constructing the plasmid pVAemp (Fig. 1). It harbors the left inverted terminal repeat, a part of the packaging signal, and the E1A gene enhancer signal of Ad5 (nucleotides [nt] 1 to 352). These sequences were derived from the plasmid pMLP10-gI, a derivative of pMLP10-gp50 (4), in which the *Eco*RI site at position 0 has been deleted. Downstream from these sequences, we cloned the 5′ part of the Ad2 VAI gene (nt 1 to 76, which includes the A and B boxes of its intragenic promoter), a polylinker, and a stop signal for RNA polymerase III transcription. These sequences were isolated by *Nsi*I cleavage from the plasmid VApIEBER, in which this region is identical to a previously described plasmid (24). The plasmid pVAr was constructed by cloning a sequence (Fig. 2), initially designed to show a potential ribozyme activity against the CAT mRNA, between the *Eco*RI and *Hin*dIII sites of pVAemp. The plasmid pVAie harbors an antisense sequence of the mRNA of the immediate-early gene of the PRV (Fig. 2), cloned between the *Sma*I and *Hin*dIII sites of pVAemp. The plasmid pVAfull was built by reconstructing a full VAI gene in pVAemp: this plasmid was cleaved by *Bam*HI, just upstream of the polylinker (Fig. 1 and 2) and *Nar*I (Fig. 1). The 3′ end of the VAI sequence was isolated by *Bam*HI and *Nhe*I cleavage from the plasmid pAdvantage (Promega) and inserted in pVAemp after the *Nar*I and *Nhe*I sites were filled in with Klenow fragment. Conformity of construction of modified VAI genes was confirmed by sequence analysis. The corresponding viruses were generated after digestion of plasmids with *Aat*II-*Acc*I (pVAemp, pVAie), *Dra*I-*Nar*I (pVAr), or *Pvu*I-*Acc*I (pVAfull) and ligation of the *Nar*I or *Acc*I end with the *Cla*I end of the large fragment of the Ad-MLP-gD genome cleaved with *Cla*I (Fig. 1). Products of the ligations were transfected into 293 cells, and then plaques were picked, expanded, and analyzed by DNA restriction analysis. The ability of Ad-VAr to drive the synthesis of the corresponding RNA was tested by

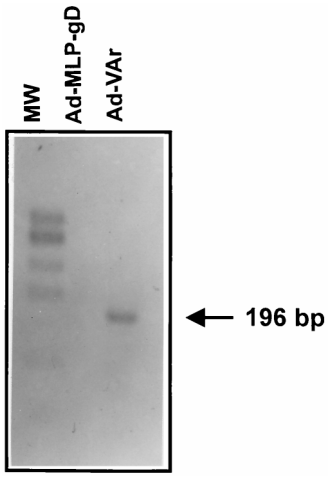

FIG. 3. Expression of VAr in adenovirus-infected cells. HeLa cells were infected with Ad-VAr or Ad-MLP-gD (Ad-gD) as negative control at a multiplicity of infection of 100 $TCID_{50}$/cell. Cells were collected 24 h p.i., and VAr RNA was amplified by RT-PCR with the primers depicted in Fig. 2. MW, molecular weight markers.

RT-PCR in HeLa cell extracts after infection with the virus (Fig. 3) with the primers depicted in Fig. 2. As expected, a positive signal corresponding to a full-length transcript (196 bp) was apparent for Ad-VAr but not for Ad-MLP-gD. No amplification products could be detected when the reverse

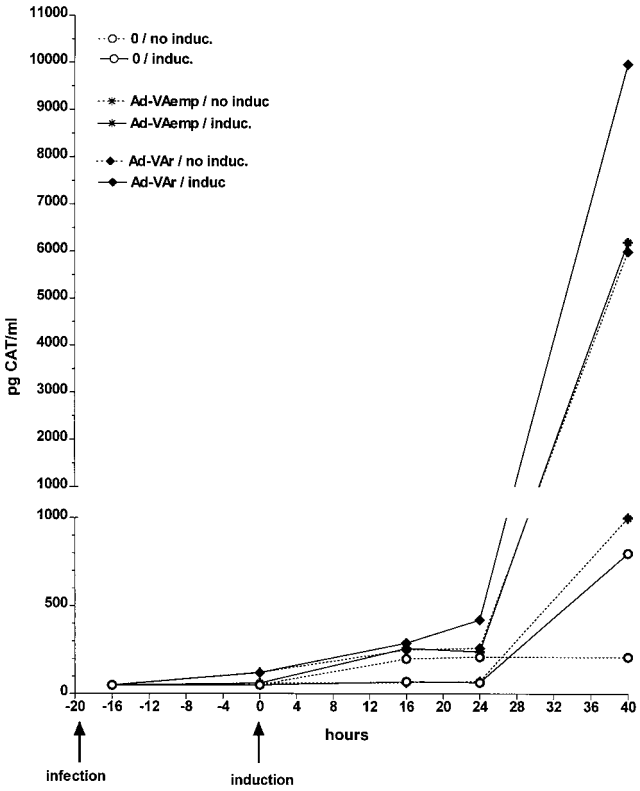

FIG. 4. A HeLa cell line expressing the CAT gene under the control of the murine metallothionein promoter was infected with Ad-VAr or AD-VAemp (100 $TCID_{50}$/cell) or left not infected. Sixteen hours p.i. (*T*0), the CAT synthesis was induced with $ZnCl_2$ (100 μM) and $CdCl_2$ (1 μM). CAT activity was quantified against time in cell extracts.

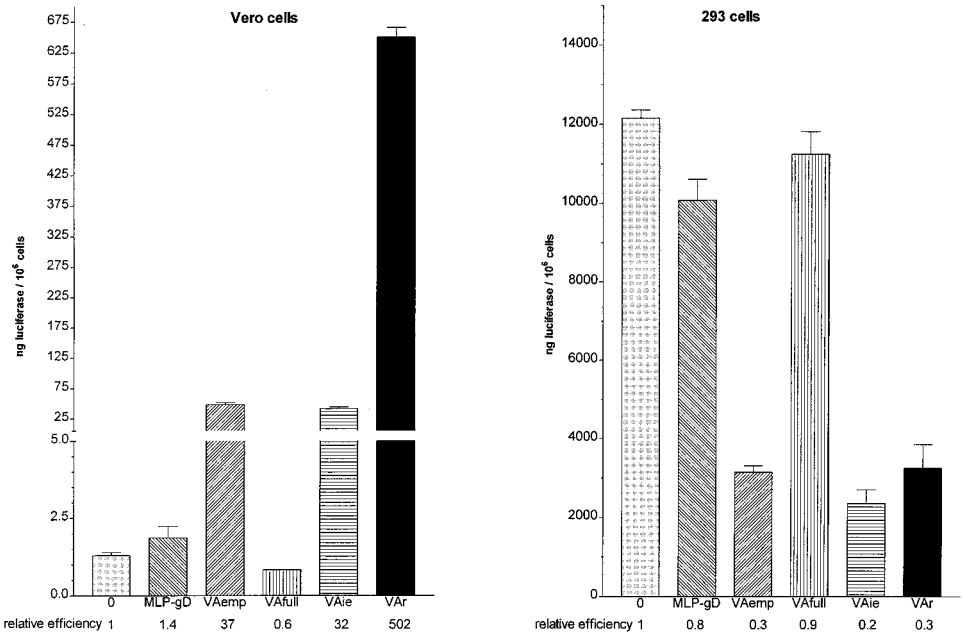

FIG. 5. Vero or 293 cells (200 to 250,000) were coinfected with Ad-MLP-luc (100 $TCID_{50}$/cell) and one of the following viruses (100 $TCID_{50}$/cell): Ad-MLP-gD (control), Ad-VAemp, Ad-VAie, Ad-VAfull, or Ad-VAr. The viruses were mixed together before infection. One batch of cells was infected only with Ad-MLP-luc. The cells were scraped 3 days later, and luciferase activity was quantified. Error bars represent standard errors of the means ($P = 0.05$) of five independent replicates.

transcription step was omitted, indicating that no false-positive amplification arose from contaminating DNA (not shown). As HeLa cells do not provide phenotypic complementation of the deleted E1A gene, except at a very high multiplicity of infection, these results demonstrated that the vector was able to drive the synthesis of detectable levels of foreign RNA under the control of the RNA polymerase III promoter in the early phase of the virus cycle.

Ad-VAr increases the level of expression of foreign genes in cell culture. As a first step, we transfected the plasmid p205mMT-CAT into HeLa cells, and after selection with hygromycin, we isolated a clone which synthesizes the CAT enzyme after induction of the murine metallothionein promoter. These cells were infected with Ad-VAr or Ad-VAemp at a multiplicity of infection of 100 50% tissue culture infective doses ($TCID_{50}$)/cell. Sixteen hours postinfection (p.i.) (*T*0), CAT synthesis was induced and quantified at 16, 24, and 40 h postinduction. Figure 4 depicts the results of one in a series of similar experiments showing convergent results. In induced cells, a 12.5-fold increase was noticed in cells infected with Ad-VAr compared to noninfected cells. In the same conditions, Ad-VAemp increased CAT synthesis by a factor of 7.5. In noninduced cells infected with Ad-VAr or Ad-VAemp, the level of CAT synthesis was increased by a factor of 29 or 5, respectively. While these observations clearly demonstrated that Ad-VAr and, less efficiently, Ad-VAemp were able to increase the synthesis of target gene products, they did not demonstrate the role of the foreign sequences cloned in the left part of the virus. In fact, these two viruses also encode an endogenous VAI gene and other viral gene products which could be responsible for this effect. Therefore, we designed a set of experiments to confirm the role of these foreign sequences.

The increase in protein synthesis depends on the sequences cloned in the left part of the virus. To confirm the role played by the modified VAr in a viral context, we conducted an experiment based on coinfection of cells with Ad-MLP-luc (which encodes its own endogenous VAI) and several adenoviruses of different genotypes. Apart from Ad-VAr and Ad-VAemp, we used (i) Ad-VAie, which harbors a modified VAI gene with a 3′ cloned sequence unrelated to that of Ad-VAr; (ii) Ad-VAfull, which harbors the native VAI gene at the left end of the genome together with its own endogenous VAI gene; and (iii) Ad-MLP-gD, which is also a virus with E1A and E3 deleted and which was included in order to study the potential contribution of endogenous VAI and E1B products encoded in *trans* and the competition for cell receptors in coinfection assays. Two cell lines were used. The 293 cells allow a full replicative cycle of all the adenoviruses tested and make high-level expression of the endogenous VAI gene possible. The Vero cells cannot support significant replication of DNA of adenovirus with E1A deleted and thus are a good model for the behavior of viruses blocked in the early phase of the virus cycle. All coinfection experiments were done at a multiplicity of infection of 100 $TCID_{50}$ per cell for each virus. Cells were collected 3 days p.i., at a time when the cytopathic effect was complete in 293 cells. The results (Fig. 5) clearly showed that modified VAI sequences were inefficient in 293 cells. On the other hand, modified VAI sequences demonstrated an inhibitory effect on the synthesis of luciferase, which can be explained by a deregulation of the phases of the virus cycle. In Vero cells, Ad-VAr had an extremely positive effect on the synthesis of luciferase (502-fold). Ad-VAemp and Ad-VAie showed a more discrete effect (37- and 32-fold, respectively). Ad-VAfull and Ad-MLP-gD had no effect on the level of luciferase synthesis. It was noteworthy that only the three adenoviruses expressing the modified VAI genes (VAemp, VAie, and VAr) demonstrated an enhancement of the expression in Vero cells but showed inhibitory effects in 293 cells. Taken together, our results show that the VAr sequence delivered into cultured cells by a replication-incompetent adenovirus very efficiently increases the level of synthesis of a target gene transduced in a similar way.

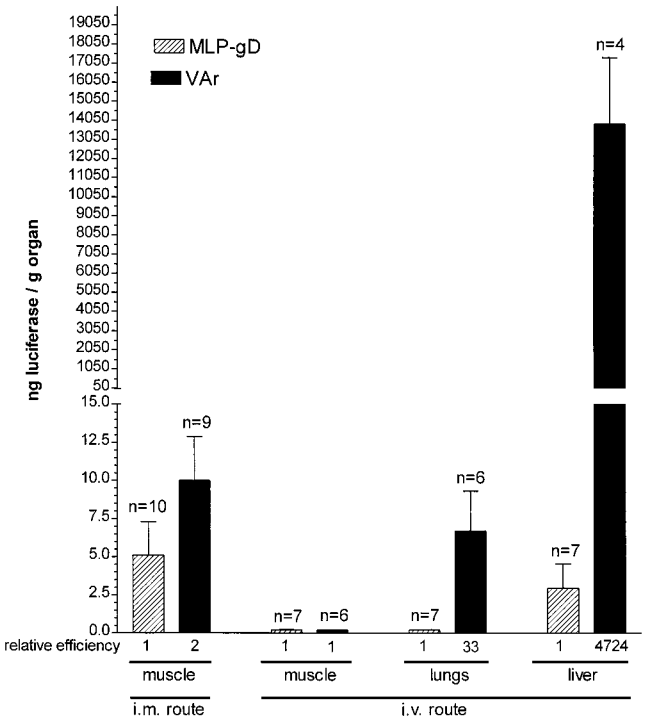

FIG. 6. Groups of 5-week-old OF1 mice were inoculated by the indicated route with Ad-MLP-luc and Ad-MLP-gD (control) or Ad-VAr. All the viruses were used at a dose of 10^9 $TCID_{50}$/mouse and were mixed together before inoculation. Organs were collected 27 h p.i. The number of mice for each point is indicated above each bar. Error bars represent standard errors of the means ($P = 0.05$).

Ad-VAr increases the level of expression of target genes in mice. We have determined whether the results demonstrated in cell culture could be extended to gene delivery into animals. All the following experiments were also conducted by coinoculation of Ad-VAr and a recombinant adenovirus expressing the gene of interest (luciferase, or the PRV gD gene for a vaccination trial).

In the first experiment, mice were inoculated by the intramuscular (i.m.) or intravenous (i.v.) route with Ad-MLP-luc plus Ad-MLP-gD (control) or Ad-VAr. The results are shown in Fig. 6. Ad-VAr induced a highly positive effect on the synthesis of luciferase in the liver (4,700-fold) and, with a lower efficiency, in the lungs (33-fold) after i.v. coinoculation. The effect was only marginal and nonsignificant in the muscle after i.m. inoculation (twofold). We hypothesized that this hierarchy reflects to a large extent the probability of infection of the same cell by a sufficient number of both adenovirus particles, which were previously shown to be maximal mainly in the liver and secondarily in the lungs after i.v. inoculation of this recombinant virus (18). On the other hand, the likelihood of infection of muscle cells is relatively low in adult mice (1). We suppose that competition between the two viruses for adenovirus cell receptors masked a possible positive effect of Ad-VAr. It is also possible that the effect of the VAr sequence was cell dependent.

We used a more sensitive approach to evaluate the efficiency of Ad-VAr injected by the muscular route, which was based on the evaluation of the immune response against the product of a foreign gene. We chose the virus Ad-MLP-gD, which harbors the PRV gD gene and is able to protect mice against a virulent PRV challenge (3, 6). We reasoned that, if the gD expression level could be increased by Ad-VAr, the protective dose of Ad-MLP-gD should be lowered. Previous experiments with mice with Ad-MLP-gD have demonstrated that the 100% protective dose by the i.m. route in mice against a standardized challenge (20 to 30 50% lethal doses by the intraperitoneal route) was 10^9 $TCID_{50}$ (3, 6). To demonstrate the efficiency of Ad-VAr, we chose lower doses of Ad-MLP-gD (10^8 and 10^7 $TCID_{50}$) and inoculated them by the i.m. route along with 10^9 $TCID_{50}$ of Ad-VAr or Ad-MLP-luc (control). The 10^9-$TCID_{50}$ dose was chosen to maximize the probability of coinfection of cells by these viruses. The results are depicted in Table 2. As expected, the virus Ad-gD with Ad-MLP-luc did not efficiently protect mice against the challenge (20% compared to 0% in control mice). The same dose injected in mice along with Ad-VAr protects 80% of the mice, which is identical to the level of production induced by Ad-gD used alone at a dose 10 times higher. A positive effect was also seen with the 10^8-$TCID_{50}$ dose of Ad-gD, but it was less obvious since a protection level of 100% was reached. Nevertheless, this effect could be identified on the antibody response (Table 2). To summarize, Ad-VAr permitted a 10-fold decrease in the protective dose of Ad-gD used by the muscular route.

TABLE 2. Efficiency of Ad-VAr for adenovirus-mediated vaccination in mice[a]

Group	Dose of Ad-MLP-gD ($TCID_{50}$)	No. of mice	Survival ratio (%)	gD antibody[b]
Ad-MLP-gD plus Ad-MLP-luc	10^7	5	20	8 ± 150
Ad-gD plus Ad-VAr			80	78 ± 150
Ad-MLP-gD plus Ad-MLP-luc	10^8	5	80	234 ± 330
Ad-MLP-gD plus Ad-VAr			100	1,136 ± 230
Control	0	5	0	ND[c]

[a] Groups of five OF1 mice were coinoculated by the i.m. route with Ad-gD at the dose indicated and with 10^9 $TCID_{50}$ of Ad-MLP-luc (control) or Ad-VAr. The viruses were mixed together before inoculation. Five weeks later, mice had blood samples taken for gD antibody testing and were challenged with 20 to 30 50% lethal doses of PRV by the intraperitoneal route. Antibodies were tested at a 1/10 dilution.

[b] Mean enzyme-linked immunosorbent assay absorbance ± standard error of the mean.

[c] ND, not done.

With the objective of demonstrating that effects seen in vivo were not restricted to transcripts originating from the MLP, we delivered Ad-VAr in mice by the i.v. route together with Ad-RSV-luc, an Ad5 with E1 and E3 deleted and expressing the luciferase gene under the control of the Rous sarcoma virus promoter. Ad-VAfull was also engaged in this experiment to see if the lack of activity of this construction observed in Vero cells could be also demonstrated in mice. The control groups were coinoculated with Ad-MLP-gD. As can be seen in Fig. 7, Ad-VAr very strongly enhanced the expression of luciferase 27 h p.i. in liver and lungs (32,300- and 128-fold, respectively), but Ad-VAfull was inefficient. All mice coinoculated with Ad-RSV-luc and Ad-VAr died between 5 and 7 days p.i. At 9 days p.i., mice from the other groups were sacrificed and luciferase activity was quantified in liver and lungs. Ad-VAfull showed no significant increase of luciferase synthesis.

DISCUSSION

A lot of work has been centered around increasing the level of expression of eucaryotic genes of interest both in cell culture

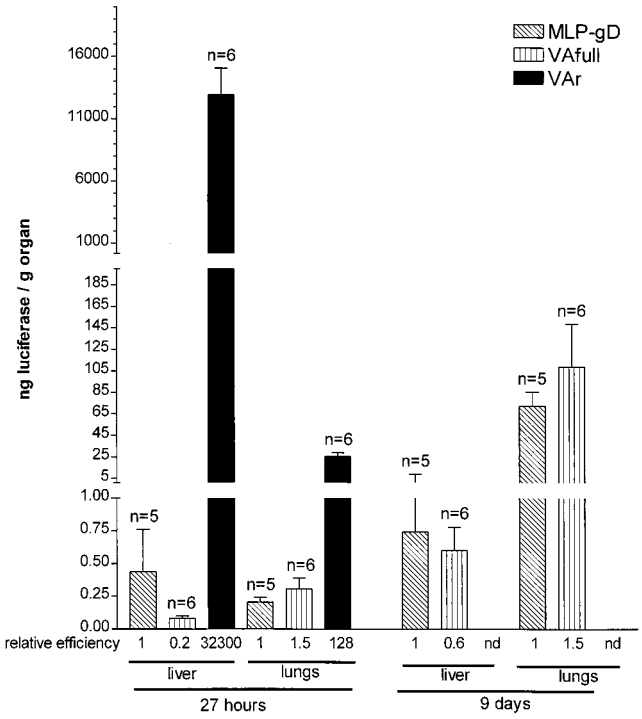

FIG. 7. Groups of 5-week-old OF1 mice were inoculated by the indicated route with Ad-RSV-luc and Ad-MLP-gD (control), Ad-VAfull, or Ad-VAr. All the viruses were used at a dose of 10^9 $TCID_{50}$/mouse and were mixed together before inoculation. Organs were collected at indicated times. The number of mice for each point is indicated above each bar. Error bars represent standard errors of the means ($P = 0.05$). nd, not done.

and in vivo. The majority of these approaches deal with the modification of *cis* sequences of the transcription unit, in order to improve transcription levels or the stability of the mRNA. We have demonstrated in this study that it is possible to increase the synthesis level of several target proteins by coexpression of the gene of interest along with a modified form of the RNA VAI delivered by infection with a replication-incompetent adenovirus. We checked whether the target gene could be present in the cell either as self-replicative extrachromosomal plasmid DNA or by being delivered by infection with a replication-defective adenovirus. This effect was verified for several target genes whose promoters, coding, and 5′ untranslated sequences differed. Different cell lines (Vero and HeLa) were tested with success. Moreover, this effect could be reproduced in vivo, in a mouse model.

We have not determined at this time whether this effect was explained by an increase in the level of transcription, stability, or translation of mRNA. To our knowledge, only one publication reports an effect of the VAI on the stability of mRNAs (21). Another study showed that the quantity of mRNAs was increased in the presence of VAI in a transient expression assay (23). Nevertheless, the clearest demonstration of the effect of the VAI was how it increased the mRNA translation level. The role of the VAI could be to favor the translation of mRNAs newly synthesized in the cell, in contrast to endogenous RNA. With this approach, the VAI could counteract the activation of the PKR induced and activated by dsRNA transcribed from plasmids or viruses used as vectors. The arrangement of the adenovirus genome implies a transcription from the two strands of the DNA and probably leads to the formation of significant amounts of dsRNAs, mainly in the late phase of the cycle. How the newly synthesized RNAs could selectively benefit from PKR inhibition is unclear, but inhibition could result in the clustering of the PKR, the newly synthesized RNAs, and the VAI in a complex, together with the translation factors. It is also possible that the VAI could selectively bind to certain classes of mRNAs.

The VAI has a complex structure. It interacts with the PKR through its apical stem, but this step does not lead to the activation of the enzyme (12, 15–17, 19). It was thought that the lack of activation resulted from the existence of insufficiently long duplexes which were not perfectly matched. In fact, the restoration of perfect matches does not activate the PKR. On the contrary, it inhibits its activation more efficiently than the wild type (5). In a second step, the central domain of the VAI binds to the PKR, which prevents its future activation. The apical stem (nt 54 to 77) is indispensable for the initial interaction with the PKR, as demonstrated by the study of deletion mutants. However, the sequence nt 54 to 76 is conserved in our constructions, and nt 77 (G) is replaced by a C. On the other hand, none of the mutants modified in their central domain inhibited the autophosphorylation of the PKR in response to dsRNAs. In the most efficient construction (Ad-VAr), the sequence nt 77 to 155 is replaced by a completely different sequence. This is also the case for Ad-VAie and Ad-VAemp, but these viruses showed a lower efficiency than Ad-VAr. It is possible that the RNA VAr possesses a secondary structure which could at least partially reproduce that of the wild-type VAI, but this is inconsistent with the fact that Ad-VAfull was clearly inefficient. So, we cannot exclude increased protein synthesis being related to other mechanisms (increase of the transcription level or stabilization of the mRNA or the ribosomal complex) (21, 23) for which the central domain of the VAI should be dispensable.

How the modified VAI sequences confer such properties on viruses which possess their own endogenous VAI is unknown. This effect is specific to the adenovirus constructions expressing the VA-derived genes, because cotransfection experiments with the corresponding plasmids gave, in the best cases, only marginal enhancement of expression (not shown). It can be related neither to a higher level of transcription in the early phase of the cycle from the VA intragenic promoter cloned just downstream of the E1A enhancer nor to an increased RNA efficiency when delivered in *trans* rather than in *cis*, because Ad-VAfull gave similar results as the control Ad-MLP-gD (Fig. 5 and 6). It can also be argued that, because Ad-MLP-luc and Ad-RSV-luc are E1A-E1B defective, the delivery of the E1B gene by all viruses with E1A deleted expressing the modified or native foreign VAI genes may have contributed to the enhancement of protein expression. In fact, the E1B 13K protein could stabilize the DNA introduced (10), having an indirect effect on the accumulation of luciferase but no positive effect on the level of transcription from the MLP (9). This hypothesis can be rejected because of the lack of activity of the control viruses Ad-MLP-gD and Ad-VAfull, which also express E1B. Another explanation could be that delivery of the E1B gene favored the transcription of the endogenous VAI transcripts, because the products of the E1A gene repress the synthesis of the VAI and the E1B 19K protein increases it (20). The direct effect of the E1B 19K protein on the transcription of the endogenous VAI gene of the luciferase-expressing viruses can be excluded because of the lack of efficacy of Ad-MLP-gD, which was used as a control because it expresses E1B (Fig. 5). Nevertheless, it is possible that the E1B 19K protein increases the transcription of the foreign VAI-derived genes. This was the reason why we did not delete it in the viruses designed to express VA-derived genes. So E1B may indirectly

participate in the effect on luciferase expression. However, this putative function could only be synergistic with that of the sequence of the modified VAI gene, because no effect could be evidenced with Ad-VAfull (Fig. 5 and 6). So, because the central domain of VAr is deleted and because VAfull lacks activity, the mechanism involved in increased protein expression is likely different from that known for the VAI.

The experiments conducted with mice showed that Ad-VAr was able to increase the luciferase level by a factor that was much higher in several organs than in cell culture. In particular, the level of expression was increased by a factor of more than 4,000 to 30,000 and 33 to 128 in the liver and the lungs, respectively, depending on the promoter used to drive the synthesis of the luciferase gene. This suggests either that the effect of VAr was more pronounced when the number of genome copies entering each cell was the factor limiting gene expression (as in vivo) or that the role of modified VAI was cell dependent. When Ad-VAr was replaced by the virus Ad-MLP-gD, the level of expression reached at 9 days p.i. was far lower than that obtained at 27 h p.i. with Ad-VAr. This shows that the effect not only concerned the time of appearance of the peak of expression but, more interestingly, modified the absolute level of expression. It must also be noted that these results paralleled those obtained with Vero cells, i.e., Ad-VAfull did not show any evidence of activity. All mice which were given Ad-VAr and Ad-RSV-luc died before day 9. Experimental coinoculation of mice and dogs with target genes other than luciferase showed that this toxicity was inconstant and likely dependent on the level of expression of the target gene, not of Ad-VAr (not shown). This is consistent either with a direct toxic effect of the accumulation of luciferase over time or with a high level of destruction of luciferase-expressing cells by the cytotoxic T-cell response. Preliminary examination of the livers of dead mice failed to reveal any evidence of inflammatory response, suggesting a direct toxic effect of the protein accumulation.

Because the target genes and the VAr genes were cloned in different viruses, the potency of such an approach has still not been optimized and the approach currently relies on the coinfection of one cell with both viruses. We are currently constructing viruses expressing these two genes within the same adenovirus genome. The insertion capacity of adenovirus vectors is not significantly limited by the shortness of VAr. As this expression of VAr does not lead to an overexpression of the target protein in 293 cells (Fig. 5), the possibility that the association of these two genes might lead to poor growth of such double-recombinant viruses seems limited. One of the main advantages of using modified VAI genes is that the transcriptional transgene control should be conserved, which is of interest for gene therapy applications. Finally, our results suggest that the potency of replication-incompetent adenovirus vectors may be enhanced both in cell culture and in vivo by coexpressing VA-derived genes together with the target gene.

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