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Individual products of the adenovirus 12S and 13S EIa mRNAs stimulate viral EIIa and EIII expression at the transcriptional level

(gene regulation/eukaryotic promoter/transcription in isolated nuclei)

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ABSTRACT **Recombinant plasmids containing mutant or wild-type adenovirus serotype 2 EIa genes that produce the 12S mRNA alone, the 13S mRNA alone, or both mRNAs were cotransfected into HeLa cells with plasmids containing the viral EIIa or EIII transcription units. The amount of RNA produced from the EIIa and EIII promoters was increased by the products of both the 13S and the 12S RNAs. By measuring the level of specific transcription in nuclei isolated from transfected cells we directly demonstrate that the increased amount of EIIa RNA is due to stimulation of the rate of transcription.**

Products of the adenovirus immediate early transcription unit EIa are required for efficient expression of the other early transcriptional units, EIb, EIIa, EIIb, EIII, and EIV (1, 2). Evidence for the role of the EIa gene comes from analysis of EIa mutant viruses (1–3) and from the use of inhibitors of protein synthesis during virus infection (4–6). These studies have suggested that products of the EIa gene may act by relieving the effect of a host repressor of adenovirus transcription (4–6) or by catalyzing the assembly of stable early transcription complexes (3). Recently, transient expression of cloned early viral genes has been studied after cotransfection into cultured cells (7–11) or microinjection into *Xenopus* oocytes (11) or cultured cells (12). The results further demonstrate the dependance of early gene expression on the presence of the EIa gene products. However, it has not been unequivocally established that the regulation takes place at the transcriptional level, since RNA transcription rates were not determined. Furthermore it is not known which of the two early EIa mRNAs, the 12S or the 13S (see Fig. 4), or both, encodes the stimulatory activity.

We have examined the effect of the EIa products on EIIa and EIII expression by cotransfecting into HeLa cells their cloned transcription units with recombinants coding for either the 12S or 13S mRNAs or for both. S1 nuclease analysis was used to measure the overall cytoplasmic accumulation of specific transcripts, and specific *in vitro* transcription in isolated nuclei was carried out to evaluate the transcription rate of the corresponding RNAs.

MATERIALS AND METHODS

HeLa cells grown in monolayer were transfected by the calcium phosphate precipitation technique as described (13) with 10–20 μg (see figure legends) of recombinant DNA per 10-cm Petri dish. After 36–60 hr, cytoplasmic RNA was purified from cells lysed with 0.3% Nonidet P-40. Nuclease S1 mapping was carried out as published (14). For transcription experiments with isolated nuclei, nuclei were prepared as described (15).

RESULTS

Accurate *in Vivo* Initiation of Transcription from Cloned EIa, EIIa, and EIII Transcription Units After Transfection into HeLa Cells. Plasmids containing the EIa, EIIa, and EIII transcription units (respectively pEIASV, pEII, and pEIII) were constructed as described in Fig. 1. These recombinant plasmids were used to transfect HeLa cells and the amount of specific RNA produced was measured by quantitative S1 nuclease analysis. Cytoplasmic RNA was isolated from HeLa cells after transfection by either pEIASV, pEII, or pEIII or 4 hr after infection by adenovirus serotype 2 (Ad2), and the RNA was hybridized to ^{32}P-5′-end-labeled DNA probes (Fig. 1) specific either for EIa, EIIa early (EIIaE), or EIII transcripts. S1 nuclease-resistant hybrids were then denatured, electrophoresed, and detected by autoradiography. The initiation sites for EIa (Fig. 2, lanes 1 and 2), EIIaE (lanes 3–5), and EIII (lanes 6 and 7) were the same in transfected cells as in the early phase of infection. The EIIaE transcription unit possesses a major start site at +1 (EIIaE1) and an additional minor one at −26 (EIIaE2) used both *in vivo* and *in vitro* (19). The locations of the EIIaE and EIII start sites (refs. 19 and 20; present results) are shown in Fig. 2. These results indicate that transcription initiates accurately from the EIa, EIIa, and EIII promoters in the transient expression assay. However, the amount of EIIa- and EIII-specific RNA is generally only 5–10% of that of EIa RNA synthesized from the same molar amounts of transfected DNA.

Stimulation of RNA Synthesis from EIIa and EIII Transcription Units by the Products of the 12S and 13S EIa mRNAs. Three overlapping mRNA species are produced by differential splicing from the EIa region. Of these, the 12S and 13S species accumulate early in infection, whereas the 9S species accumulates only during the late phase. The stimulatory activity of EIa must therefore be encoded by either the 12S or 13S mRNA or by both. To examine the splicing pattern of the EIa transcripts under our experimental conditions, cytoplasmic RNA from cells transfected with pEIASV was analyzed under denaturing conditions and compared with late Ad2-infected cell RNA (Fig. 3, lanes 1 and 3). Both the 12S and 13S species are made in the EIa-transfected cells (lane 3) but the 13S product accumulates about 4 times more efficiently.

To determine the effects of the separate EIa products on the levels of EIIa and EIII expression, pEII and pEIII were cotransfected with either the recombinant containing the EIA transcription unit (pEIASV) or intron-less derivatives of this plasmid producing either the 13S (pEIA13S) or the 12S (pEIA12S) mRNAs (see Fig. 4 for construction details).

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Abbreviations: Ad2 and Ad5, adenovirus types 2 and 5.

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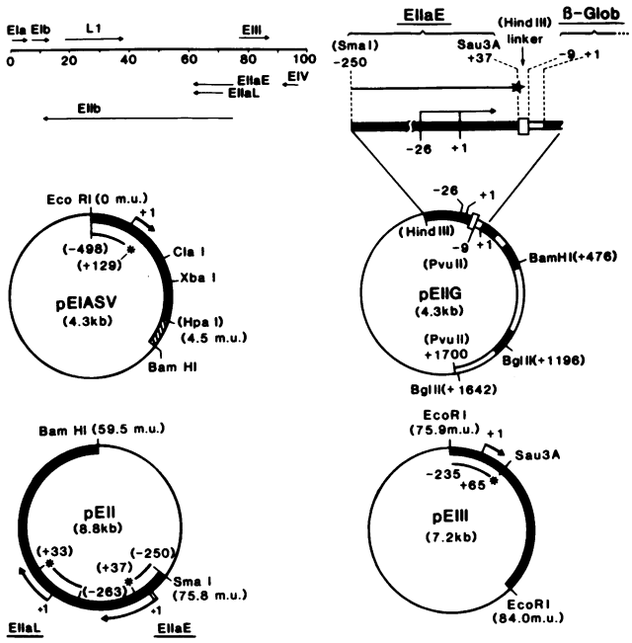

FIG. 1. Organization of early adenovirus transcription units and construction of pEIASV, pEII, pEIIG, and pEIII recombinants. A genomic map of adenovirus DNA showing early viral transcription units and the EIIaL transcription unit is presented (taken from data in ref. 16). pEIASV contains the left 4.5% of the Ad2 genome (*Hpa* I fragment) ligated to the 135-base-pair-long simian virus 40 (SV40) *Hpa* I (2604)/*Bam*HI (2469) fragment containing the SV40 early and late polyadenylylation signals. The *Hpa* I restriction site at the junction was lost during the construction. This fragment was inserted between the *Eco*RI and *Bam*HI sites of pML2, a derivative of pBR322 (17). The DNA probe used for S1 nuclease mapping is the coding strand of the *Sau*3A/*Eco*RI fragment (+129 to −498 with respect to the EIa cap site) labeled with ^{32}P at the 5′ end. pEII is the Ad2 *Bam*HI/*Sma* I fragment [59.5 map units (m.u.) to 75.8 m.u.] inserted between the *Bam*HI and *Sma* I sites of pACYC177 (18). Both the early (EIIaE) and late (EIIaL) promoters are present on this plasmid. The EIIaE probe is the coding strand of the *Sau*3A/*Sma* I fragment (+37 to −250 with respect to the EIIaE major cap site) labeled at the 5′ end. The EIIaL probe extending from the *Fnu*DII site at +33 to the *Fnu*DII site at −263 used in some experiments is also shown. pEIII is the Ad2 *Eco*RI D fragment (75.9–84.0 m.u.) inserted into the *Eco*RI site of pBR322 and contains the EIII transcription unit from position −237 to +2440. The EIII probe is the coding strand of the *Sau*3A/*Eco*RI fragment labeled with ^{32}P at the 5′ end (+65 to −235 with respect to the EIII cap site). pEIIG is a 287-base-pair-long fragment of the EII promoter (extending from position −250 to position +37, with respect to the EIIaE1 cap site; black boxes) linked by a *Hin*dIII linker (open vertical box) to the rabbit β-globin *Pvu* II fragment extending from −9 to about +1700 (with respect to the globin cap site; open box, with exons hatched) inserted between the *Hin*dIII and *Pvu* II sites of pBR322 (thin line). Transcripts initiated from the EIIa promoter were detected by hybridization to the EIIaE probe (*Sma* I/*Sau*3A fragment) shown in the expanded region and identical to that shown on the pEII recombinant. Restriction enzyme sites in parentheses were lost during construction. Arrows indicate the direction of transcription. Asterisks mark the labeled 5′ ends of the probes used for S1 mapping. kb, Kilobase pairs.

Cytoplasmic RNA was isolated from cells 48 hr after transfection, and the amount of specific (EIa, EIIa, or EIII) RNA was measured by quantitative S1 nuclease analysis. Fig. 5*A*, lane 1, shows the basal level of EIIa-specific RNA in HeLa cells transfected with the EIIa recombinant (pEII) alone. When pEIASV was cotransfected with pEII, an increase (≈20-fold) in the level of EIIa-specific RNA was observed (Fig. 5*A*, lane 2). A similar stimulation of EIIa expression was also observed when pEII was cotransfected with

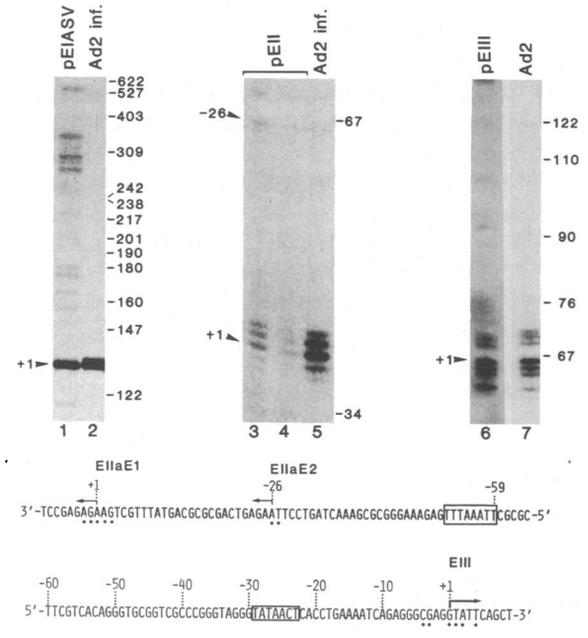

FIG. 2. **Specific initiation of transcription from the adenovirus EIa, EIIa, and EIII promoters in adenovirus-infected cells and in cells transfected with cloned viral genes.** Cytoplasmic RNA isolated from a suspension culture of Ad2-infected HeLa cells 4 hr after infection (19) was analyzed by S1 nuclease protection using the EIa (lane 2), EIIaE (lane 5), and EIII (lane 7) probes (Fig. 1). Quantitative S1 nuclease analysis of cytoplasmic RNA isolated from HeLa cells transfected with 15 μg of pEIASV (lane 1), pEII (lane 3), or pEIII (lane 6) or 10 μg of pEII (lane 4) was carried out using the same probes. Arrowheads point to probe fragments protected by specific transcripts discussed in the text. Sequences of the EIIaE and EIII promoter regions are shown below. RNA start sites used in lanes 3–7 are indicated by dots under the sequences and nucleotides labeled +1 (EIIaE1 and EIII) and −26 (EIIaE2) refer to the major start sites already determined (19–21). The "TATA"-like sequences at position −59 (EIIaE) and −29 (EIII) are boxed. Positions of DNA size markers (*Msp* I fragments of ^{32}P-end-labeled pBR322) are indicated. The autoradiograms shown in lanes 3–6 were exposed several times longer than those shown in lanes 1 and 2.

pEIA13S or pEIA12S (lanes 3 and 4). Expression from both the EIIaE1 (+1) and EIIaE2 (−26) start sites was stimulated. The stimulation varied between 10- and 40-fold in various experiments using several independent plasmid preparations. The amount of EIa-specific cytoplasmic RNA was essentially constant in all experiments (Fig. 5*A*, bands marked EIa +1). Late in infection, transcription from the EIIa gene also proceeds from a promoter (EIIaL, Fig. 1) located about 1 kilobase downstream from the EIIaE promoter. We attempted to measure the effect of EIa expression on this promoter, using the EIIaL probe shown in Fig. 1, but did not detect any cytoplasmic EIIaL-specific RNA in the absence or presence of cotransfected EIA plasmids (results not shown).

To examine the effect of EIa products on expression of the EIII transcription unit, cotransfection experiments were performed with pEIII and either pEIASV, pEIA12S, or pEIA13S. These experiments (Fig. 5*B*) revealed a stimulatory pattern similar to that observed for the EIIa transcription unit. Cotransfection with pEIASV, pEIA12S, or pEIA13S stimulated the level of RNA produced from the EIII plasmid by at least 10-fold (compare lane 1 with lanes 2–4). Stimulation of EIII expression by the 12S and 13S RNA products (lanes 3 and 4) is at least as high as that obtained with pEIASV (lane 2), if the level of EIII transcripts is normalized according to the amount of EIa-specific RNA (band EIa +1 in lanes 2, 3, and 4).

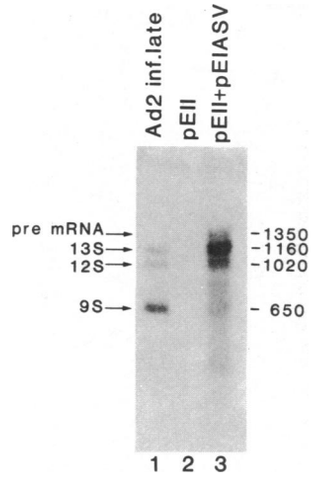

FIG. 3. Size analysis of cytoplasmic EIa mRNAs. Cytoplasmic RNA was isolated from a suspension culture of Ad2-infected HeLa cells (19) 18 hr after infection (lane 1) and from HeLa cells 60 hr after transfection with 10 μg of pEII alone (lane 2) or cotransfection with 10 μg of pEII and 10 μg of pEIASV (lane 3). The RNA ($\approx$30 μg per lane) was denatured, electrophoresed on a methylmercury hydroxide/agarose (2%) slab gel and transferred to diazobenzyloxymethyl-paper as described (22). EIa-specific RNA was visualized by hybridization to the *Eco*RI/*Xba* I fragment of pEIASV (Fig. 1), ^{32}P-labeled by nick-translation. The specificity of this probe is confirmed by the absence of hybridization to RNA present in lane 2. Numbers on the right correspond to length of nucleotides (nt) of the corresponding RNA molecules as deduced from the positions of single-stranded DNA size markers run on the same gel. Arrows on the left indicate expected position of the EIa precursor RNA [1131 nt + poly(A)], 13S mRNA [1016 nt + poly(A)], 12S mRNA [878 nt + poly(A)], and 9S [541 nt + poly(A)], respectively (23). The poly(A) tails were assumed to be 100–200 nt long.

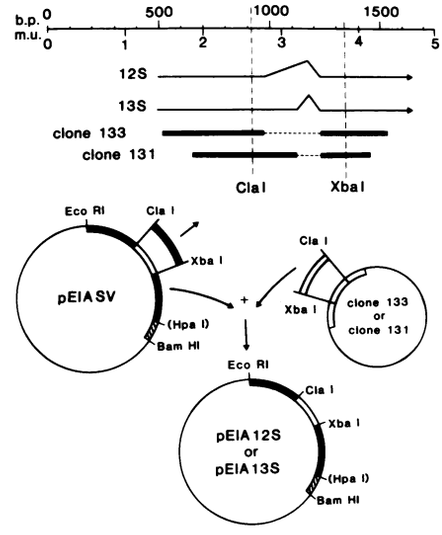

FIG. 4. Organization of the EIa transcription unit and construction of intron-less plasmids coding for 13S or 12S EIa RNAs. A map of the left-terminal 5% of the Ad2 genome showing the structure of the early 12S and 13S mRNA species encoded by the EIa transcription unit (taken from data in ref. 16) is presented. The direction of transcription is indicated by arrows and the introns, by carets. The sequences present in the corresponding cDNA clones 133 and 131 of Perricaudet *et al.* (24) are shown below (thick bars), with the absent sequences indicated by dotted lines. pEIA12S and pEIA13S recombinants were constructed by replacing the *Cla* I/*Xba* I fragment of pEIASV by the *Cla* I/*Xba* I fragment of clone 133 or clone 131.

Stimulation of EIIa Expression by EIa Gene Products Occurs at the Level of Transcription. To determine whether the increased accumulation of EIIa cytoplasmic RNA was due to an increased rate of transcription or to a post-transcriptional event, we compared the amount of specific EIIa cytoplasmic RNA with the actual transcription rate in the same series of transfection experiments (Fig. 6 and Table 1). In these experiments, a plasmid containing the rabbit β-globin coding sequences under the transcriptional control of the EIIa promoter (pEIIG, Fig. 1) was used. Transcription of pEIIG in transfected HeLa cells was initiated from the same sites of the EIIa promoter as in pEII (compare Fig. 6*A* with Fig. 5*A*). Cotransfection of pEIASV, pEIA12S, or pEIA13S with pEIIG produced a pattern of stimulation similar to that obtained with pEII (compare Figs. 5*A* and 6*A*). This indicates that the stimulation of EIIa transcription is dependent only on sequences located upstream from position +37. EIIG cytoplasmic RNA accumulation (Table 1), measured by quantitative S1 nuclease analysis was, in this particular experiment, increased between 6- and 10-fold in the presence of recombinants bearing the complete EIa transcription unit (pEIASV) or its 12S and 13S RNA-producing counterparts (pEIA12S and pEIA13S). In several independent experiments, the level of stimulation varied between 5- and 20-fold, probably because of differences in transfection efficiencies. However, in any given experiment, measurements of nuclear plasmid copy number have shown that the transfection efficiency of the various cotransfected recombinants did not vary by >2-fold (data not shown).

The effect of EIa cotransfection on the actual rate of transcription from the EIIa promoter was estimated by measuring the amount of labeled RNA transcribed from EIIa sequences in nuclei isolated from the cells used for the cytoplasmic RNA analysis shown in Fig. 6*A*. Transcription was carried out under conditions in which re-initiation by free RNA polymerase molecules is prevented, allowing only the elongation of transcripts initiated *in vivo* (29). Thus, the amount of radioactivity incorporated into EIIG-specific RNA is an indication of the number of RNA polymerase molecules that were actually engaged in transcription of EIIa sequences *in vivo*. The amount of EIIG-specific transcription was quantitated by hybridization to an excess of the coding strand of the EIIG transcription unit bound to a nitrocellulose filter. The results [Fig. 6*B* (row b, columns 1–6) and Table 1] show that the relative level of stimulation of EIIa-specific cytoplasmic RNA by the EIa products (Fig. 6*A*, lanes 1–6) is reflected in the relative level of EIIG-specific nuclear transcription. From these results, we conclude that the EIa-mediated stimulation of the EIIa expression is due to an increase in the rate of EIIa transcription rather than to stabilization of RNA or any other post-transcriptional event. The transcription rate of pEIIG was repeatedly higher when cotransfection was with pEIA12S or pEIA13S than when it was with pEIASV or the combination of pEIA12S and pEIA13S, while the transcription rate of the pEIA series did not vary significantly (Table 1). The rate of nuclear transcription was also measured for the noncoding strand of pEIIG (Fig. 6*B*, row c). The results indicate that EIa products also stimulated transcription on this strand but to a lesser extent (Table 1 and *Discussion*). Cotransfection of pEIIG with a recombinant (pEIA$^-$) containing the whole 5′ region of the EIa transcription unit (from position −498 to position +129; Fig. 6*A*), but not the EIa structural sequence, did not increase (over constitutive levels) the rate of EIIG transcription or the amount of EIIG-specific cytoplasmic RNA [Fig. 6*A* and *B* (lane 2) and Table 1]. Since pEIA$^-$ contains the entire EIa promoter region (25), these results rule out the possibility that the stimulatory effect of cotransfection with pEIASV could be due to a recombination event resulting in the insertion of an EIa enhancer element (25, 30, 31) into the EIIa plasmid rather than to a *trans* effect of the EIa products. Note that, although pEIA$^-$ was transcribed at

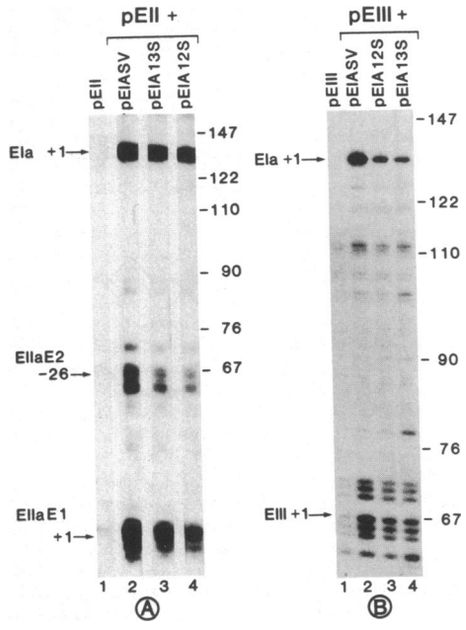

FIG. 5. Induction of EIIa and EIII expression by EIa products in transfected HeLa cells. (*A*) Quantitative S1 nuclease analysis of cytoplasmic RNA isolated from HeLa cells 60 hr after transfection with 10 μg of pEII alone (lane 1) or cotransfection with 10 μg of pEII and 10 μg of either pEIASV (lane 2), pEIA13S (lane 3), or pEIA12S (lane 4). (*B*) Quantitative S1 nuclease mapping of cytoplasmic RNA isolated from HeLa cells 60 hr after transfection with 15 μg of pEIII alone (lane 1) or 15 μg of pEIII and 5 μg of either pEIASV (lane 2), pEIA12S (lane 3), or pEIA13S (lane 4). Probe fragments protected by specific transcripts and positions of DNA size markers are indicated as in Fig. 2.

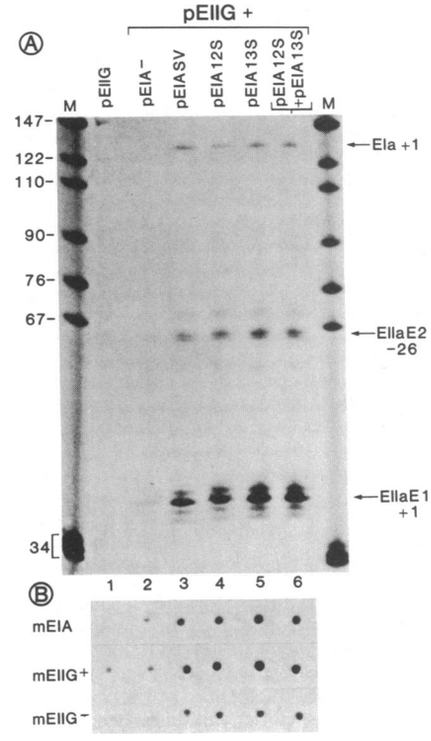

FIG. 6. Induction of transcription from the EIIa promoter by EIa gene products. (*A*) Cytoplasmic RNA isolated from HeLa cells 36 hr after transfection with 5 μg of pEIIG alone (lane 1) or together with 5 μg of pEIA⁻ (lane 2), pEIASV (lane 3), pEIA12S (lane 4), or pEIA13S (lane 5) or 2.5 μg each of pEIA12S and pEIA13S (lane 6). EIIaE1 (+1) and EIIaE2 (−26) starts are indicated as in Fig. 5*A*. Recombinants pEIIG, pEIASV, pEIA12S, and pEIA13S are described in Figs. 1 and 4. Recombinant pEIA⁻ is derived from pEIASV by deleting the coding sequence between positions +129 and +1075 (25). The probes were the same as those used for the experiment shown in Fig. 5*A* (described in Fig. 1). The DNA size markers shown in lane M are the same as in Fig. 2. (*B*) Transcription from the EIIaE promoter in isolated nuclei prepared from HeLa cells transfected with pEIIG alone (column 1) or together with pEIA⁻ (column 2), pEIASV (column 3), pEIA12S (column 4), or pEIA13S (column 5) or a mixture of pEIA12S and pEIA13S (column 6). Nuclei were prepared from the transfected cells used for the cytoplasmic RNA analysis shown in *A*. Labeled RNA was prepared by incubating an aliquot of nuclei (corresponding to ≈20 μg of DNA) for 30 min at 32°C in an 80-μl reaction mixture containing 10% glycerol (vol/vol), 40 mM Tris·HCl (pH 7.9), 350 mM ammonium sulfate, 1.25 mM dithiothreitol, 10 mM $MgCl_2$, heparin sulfate at 1 mg/ml, 0.5% Sarkosyl, 0.5 mM GTP/ATP/UTP, and 2 μM [α-^{32}P]CTP (410 Ci/mmol). RNA was purified essentially as described (15), except that the first phenol/chloroform extraction was omitted. The purified RNA was hybridized to dot blots containing the following single-stranded DNA probes: the coding strand of the 12S cDNA insert of ≈840 nucleotides (see ref. 24) of clone 133 shown in Fig. 4, cloned into the *Pst* I site of M13mp8 (mEIA, row a); the coding and noncoding strands of the *Bam*HI/*Bgl* II fragment of pEIIG (extending from position +476 to position +1196 in the globin coding region; Fig. 1), cloned into the *Bam*HI site of M13mp8 (mEIIG⁺ and mEIIG⁻, respectively, rows b and c). Single-stranded M13 phage recombinant DNA was prepared as described (26) with an additional hydroxyapatite purification step (27). Nitrocellulose filters (Schleicher & Schüll) were spotted with 2 μg of single-stranded DNA and treated as described (28). Hybridization and filter washing conditions were as described (28) except that dextran sulfate was omitted from the hybridization reaction and an RNase A treatment (10 μg/ml in 0.3 M NaCl/0.03 M Na citrate, pH 7, at 37°C for 30 min) was included during the washing procedure. Typically, nuclear transcription yielded ≈5 × 10^6 cpm of total labeled RNA of which 2 × 10^6 cpm was used for dot blot hybridization.

least as efficiently as the other pEIA plasmids, it produced only one-tenth the amount of cytoplasmic RNA (Table 1).

DISCUSSION

By using recombinants that produce either the 13S or 12S EIa mRNA species, we have demonstrated that the amount of RNA produced from the EIIa and EIII promoters is stimulated by the individual EIa products. The stimulation by the 13S RNA product is consistent with the results of Montell *et al.* (32), who found that early RNA synthesis was normal during infection with a mutant defective in 12S RNA production. Previous work has not provided a clear picture of the role of the 12S mRNA product in the induction of early viral gene expression. It was found that an adenovirus serotype 5 (Ad5) single-base deletion mutant (33), H5hr1, producing a defective 13S, but a normal 12S, EIa mRNA did not synthesize any other early transcripts (2). However, a similar frame-shift mutant, H5in500, affecting only the 13S mRNA product (34), produced nearly wild-type levels of EIb, EIIa, and EIII transcripts. Our present results, which show conclusively that RNA production from the EIIa and EIII transcription units can be efficiently stimulated on cotransfection with the pEIA12S recombinant that codes only for the 12S RNA, are in agreement with this latter result.

By using pulse-labeling techniques, Nevins (4) has shown that the transcription rates of all early genes are depressed in cells infected with EIa-defective mutants (dl312 and H5hr1), suggesting that EIa may act at the level of transcription. On the other hand, Persson *et al.* (5) have proposed that EIa products increase the stability of early RNAs. The quantitative S1 nuclease assay reported here gives a precise measurement of specific cytoplasmic RNA accumulation in the transfected cells rather than a direct measurement of promoter activity. Variations in the accumulation of EIIa and

Table 1. Stimulation of EIIa transcription by EIa recombinants

	pEIIG	pEIIG + pEIA$^-$	pEIIG + pEIASV	pEIIG + pEIA12S	pEIIG + pEIA13S	pEIIG + pEIA12S + pEIA13S
Relative amount of specific cytoplasmic RNA produced						
pEIA series	0	0.1	1	0.5	0.9	0.8
pEIIG	1	0.8	5.9	7.6	10.3	9.9
Relative transcription rates in isolated nuclei						
pEIA series (coding strand)	0	3.7	1	2	1.2	1.4
pEIIG coding strand	1	1.7	5.4	10.7	17.9	6.1
pEIIG noncoding strand	0.7	0.7	1.7	1.9	2	2.2

Amounts of specific transcripts were measured by determining the radioactivity present in the protected probe fragments marked EIa +1, EIIaE1, and EIIaE2 for each transfection (lanes 1–6 in Fig. 6*A*). cpm for EIIaE1 and EIIaE2 were combined. Amounts of EIa transcripts are presented relative to the EIa level in lane 3 and those of EIIa are relative to the EII level in lane 1 (Fig. 6*A*). The nuclear transcription rates on the pEIASV derivatives were deduced from the amount of labeled RNA hybridized in each spot of row a of Fig. 6*B*. cpm for pEIA$^-$ (column 2, Fig. 6*B*) were normalized to the 840-nucleotide length of the 12S cDNA insert of the mEIA probe (see legend to Fig. 6), assuming that all pEIA$^-$ transcripts hybridize to the first 129 nucleotides of that cDNA sequence and that all pEIASV, pEIA13S, and pEIA12S transcripts hybridize to the whole cDNA insert. The transcription rates on both strands of pEIIG were estimated from the amount of labeled RNA hybridized to the coding and noncoding strands of the globin probe (rows b and c, respectively, Fig. 6*B*). cpm hybridized to identical amounts of single-stranded M13mp8 DNA present in the same hybridization reactions (data not shown) were taken as the background level and subtracted. The final figures of transcription rates were expressed, for the pEIA series, relative to the values found for pEIASV and, for pEIIG, relative to the value found for the coding strand of pEIIG transfected alone.

EIII transcripts could be the result of variations in promoter efficiency as well as in RNA processing and stability or a combination of both. Similarly, assays measuring accumulation of the protein products encoded by the EIIa gene (10, 12) or by chimeric genes (7, 11) are also indirect assays of promoter function. Our *in vitro* nuclear transcription data (Fig. 6, Table 1) definitely establish that the stimulation of EIIa expression by the EIa products is due to an increase in the rate of transcription. The same nuclear transcription experiments (Table 1) also indicate a significant stimulation of synthesis from the noncoding strand of the EIIa plasmid, pEIIG. This could be due to activation of cryptic "substitute" promoter elements located in this strand. This stimulation is reminiscent of observations showing that the EIa products can stimulate RNA synthesis from a variety of nonviral promoter elements, such as the human heat shock (35) and β-globin (36) promoters. Further studies will establish whether the activation of the adenovirus early transcription units and of other natural or substitute promoters by the EIa products have the same molecular basis.

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