Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 7132–7136, December 1982
Biochemistry

Sequences upstream from the T-A-T-A box are required *in vivo* and *in vitro* for efficient transcription from the adenovirus serotype 2 major late promoter

[RNA polymerase (II)/microinjection in nuclei/HeLa cell transfection/circular DNA]

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Communicated by Gary Felsenfeld, August 20, 1982

ABSTRACT **We show that sequences located upstream from the T-A-T-A box, between positions −97 and −34, are necessary for efficient *in vivo* transcription from the adenovirus serotype 2 major late promoter. The effect of these upstream sequences was also investigated *in vitro* using a whole cell or an S100 extract and circular or linear templates. With the whole cell extract, the *in vivo* effect of the upstream sequences was reproduced *in vitro*. With the S100 extract, some effect of the upstream sequences was observed with circular, but not with linear, templates.**

The comparison of DNA sequences in the 5′ flanking regions of genes coding for proteins has revealed two specific regions of homology. The highly conserved T-A-T-A box sequence, located about 30 nucleotides upstream from the cap site, is a promoter element required for efficient and selective transcription both *in vitro* and *in vivo* (see refs. 1 and 2 for review). A second region of homology, located at a variable position upstream from the T-A-T-A box, appears to be less conserved (3, 4) and is necessary for efficient *in vivo* transcription (5–10). The *in vitro* effect of these further upstream sequences is less clear: in some cases, they had no or little effect (1, 11–15) while, in others, they appeared to be important for efficient transcription (16–19). We show here that these upstream sequences are required both *in vivo* and *in vitro* for efficient transcription from the adenovirus serotype-2 major late promoter.

MATERIALS AND METHODS

HeLa cells (20–40% confluence) were transfected as described (20) with 20 μg of recombinant DNA per 10-cm Petri dish and 2 μg of the β-globin plasmid pβ(244+)β (21) as an internal control. After 48 hr, cytoplasmic RNA was purified from cells lysed with 0.3% Nonidet P-40. For nuclease S1 mapping, RNA from *in vivo* (50 μg, about one Petri dish) and *in vitro* assays was dissolved in 10 μl of 10 mM Pipes, pH 6.5/0.4 M NaCl containing an excess of the *Hin*dIII/*Xho* I probe (see Figs. 1*B* and 2) and the internal control probe when indicated, heated 5 min at 85°C, and hybridized at 68°C for 12 hr. After digestion with nuclease S1 (11), the resistant DNA fragments were analyzed on 8% acrylamide/8.3 M urea gels (22). Figs. 2–5 represent typical experiments.

RESULTS

Microinjection in CV1 Cell Nuclei of Recombinants Containing the Ad2 Major Late Promoter (Ad2MLP) Shows that Sequences Upstream from the T-A-T-A Box Are Essential for Efficient *in Vivo* Expression. A series of Ad2MLP mutants deleted to positions −200, −150, −97, −62, −34, −32, −29, and −21 (see Fig. 1 and ref. 1) was linked to simian virus 40 (SV40) tumor-antigen coding sequence (pSVA series, Fig. 1). After microinjection of the recombinants into CV1 cell nuclei, tumor-antigen accumulation was used to estimate Ad2MLP activity. The same level of expression was obtained with wild-type pSVA500 and with pSVA200, -150, and -97 (Table 1). No decrease in tumor-antigen expression was observed when the sequences between −500 and −260 were deleted (not shown). However, tumor-antigen expression was significantly decreased in pSVA34 and pSVA32 (Table 1), both of which have an intact T-A-T-A box (Fig. 1). This effect is more pronounced at the lower DNA concentration, which is likely to better reflect the relative promoter efficiency of the deletion mutants. Deletion of the T-A-T-A box (pSVA21) or of its first two bases (pSVA29) resulted in a further decrease, but the values were still significantly higher than those obtained with pEMP, which has no promoter sequence. From these results, it appears that both the T-A-T-A box and a region located further upstream, between positions −97 and −34, are important for the *in vivo* activity of the Ad2MLP.

Insertion of the SV40 72-BP Repeat in the pSVA Recombinants Allows Detection of Specific Transcription from the Ad2ML Promoter. To analyze the effect of deletions in the Ad2MLP region at the transcriptional level, we switched to HeLa cells because their transfection efficiency is higher than that of CV1 cells. We quantitated the amount of Ad2ML-specific RNA by nuclease S1 mapping. With pSVA500 (not shown) or pSVA34 (Fig. 2*B*, lane 1), a band corresponding to RNA initiated from the Ad2MLP (position MLP+1) could not be detected. This observation prompted us to investigate whether the SV40 72-bp repeat, known to enhance gene expression (20, 24), could be used to increase RNA transcription from the Ad2MLP. Transfection into HeLa cells of pSVBA34 and pSVBA29 (Fig. 2*A*), derived from pSVA34 and -29 by insertion of the 72-bp repeat close to the T-A-T-A box, resulted in a dramatic increase of Ad2MLP-specific RNA (lanes 2 and 8). As previously reported (24), insertion of the 72-bp repeat in the opposite orientation (pSVBIA34 and -29) did not affect the magnitude of its effect whereas deletions within the 72-bp repeat (see legend to Fig. 2), known to reduce its effect (24), resulted in a striking decrease in the amount of specific RNA (compare lane 2 with lanes 4 and 5).

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Abbreviations: Ad2 and Ad5, adenovirus serotypes 2 and 5; SV40, simian virus 40; Ad2MLP, Ad2 major late promoter; bp, base pair(s); kb, kilobases.

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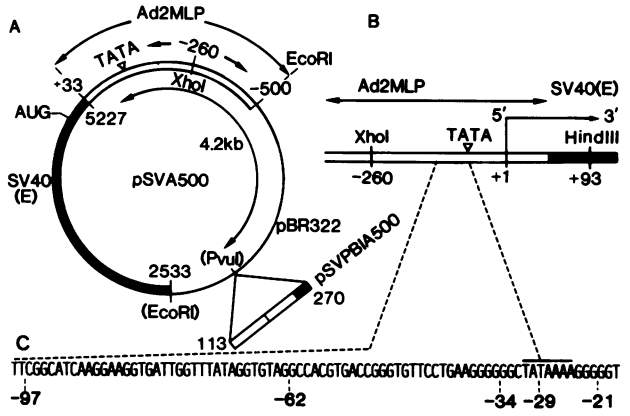

FIG. 1. Construction of the pSVA series. (*A*) pSVA plasmids were constructed by recombination of parts of plasmids pEMP (23) and pMLA (1). pEMP contains the SV40 tumor-antigen amino acid coding sequence (coordinates 2,533–5,227) (24) inserted between the *Eco*RI and the *Bam*HI sites of pBR322 (sites shown in parentheses were lost during the constructions). pMLA contains the Ad2 *Bal* I E fragment inserted into the *Eco*RI site of pBR322. Deletions in the Ad2MLP region were created (arrows) from the unique −260 *Xho* I site (1). The wild type or deletion-containing fragments were excised with *Sal* I (position 650 in pBR322) and *Pvu* II (position +33 in the Ad2MLP) and inserted between the repaired *Bam*HI and the nonrepaired *Sal* I sites of pEMP. pSVA500 contains the wild-type Ad2MLP region from −500 to +33 (double line) inserted upstream from the SV40 early [SV40 (E)] coding sequences (heavy line). Recombinants pSVA200, -150, -97, -62, -34, -32, -29, and -21 contain 200, 150, 97, 62, 34, 32, 29, and 21 base pairs (bp) upstream from the initiation site (+1), respectively, as determined by sequence analysis (pSVA97, -62, -34, -32, -29, and -21) or restriction enzyme mapping (pSVA500, -200, and -150). pSVPBIA500, -150, -97, -62, -34, -29, and -21 correspond to the pSVA series in which a SV40 DNA fragment (coordinates 113–270, see ref. 24) containing the 72-bp repeat was inserted in the *Pvu* I site of pBR322 in the orientation opposite to that of the SV40(E) sequence. The direct repeat is represented by the open boxes and a flanking segment is represented by the closed box. (*B*) Structure of the boundary between the Ad2MLP (double line) and the SV40(E) region (heavy line) in pSVA500. (*C*) Nucleotide sequence of the noncoding strand of the Ad2MLP region (25) from positions −99 to −19.

pSVBA29 and pSVBIA29, which lack the first two bases of the T-A-T-A box, were almost as efficient in promoting specific transcription from the Ad2MLP as pSVBA34 and pSVBIA34, which possess an intact T-A-T-A box (compare lanes 2 and 3 with lanes 8 and 9). This observation, in apparent contradiction with

Table 1. Microinjection of pSVA recombinants in CV1 cell nuclei

Recombinant	% immunofluorescent CV1 cells	
	100 μg of DNA/ml	33 μg of DNA/ml
pSVA500	100 (6)	100 (2)
pSVA200	89 (3)	140 (2)
pSVA150	95 (3)	127 (2)
pSVA97	110 (3)	138 (2)
pSVA34	32 (6)	6 (2)
pSVA32	31 (2)	5 (2)
pSVA29	7 (6)	0 (2)
pSVA21	9 (3)	0 (2)
pEMP	0 (6)	0 (2)

About 20 fl of recombinant DNA at 33 or 100 μg/ml was injected per CV1 cell nucleus as described (24). pEMP and the pSVA series are described in Fig. 1. The results were expressed as the percentage of injected cells that were positive for tumor antigen, taking as 100% the value obtained with pSVA500. Values in parentheses correspond to the number of independent assays carried out for each recombinant. For convenience, we microinjected CV1 cells but similar results (not shown) were obtained with HeLa cells.

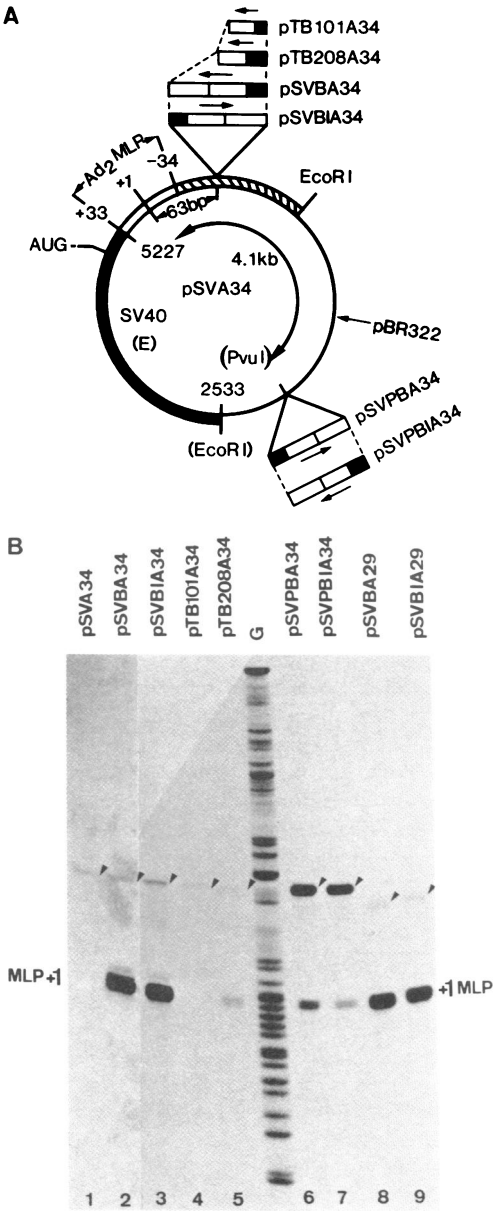

FIG. 2. The SV40 72-bp repeat enhances specific *in vivo* transcription from the Ad2MLP. (*A*) Construction of derivatives of pSVA34 and pSVA29 containing the wild-type or mutated 72-bp repeat. pSVA34 and pSVA29 were constructed as described in Fig. 1. (Only pSVA34 is represented and the replacing adenovirus sequences upstream from position −34 are hatched.) The segment containing the 72-bp repeat (see Fig. 1*A*) was then inserted in both orientations [the arrows indicate the natural orientation with respect to the SV40(E) sequence] at 63 bp (*Sst* I site; see ref. 24) (pSVBA34 and pSVBIA34) or at 4.1 kilobases (kb) (pSVPBA34 and pSVPBIA34) from the Ad2ML cap site. Two other recombinants (pTB101A34 and pTB208A34) contain the fraction of the 72-bp repeat present in the deletion mutants TB101 and TB208 previously described (24). pSVBA29 and pSVBIA29 were constructed as pSVBA34 and pSVBIA34. (*B*) Nuclease S1 mapping of the 5′ ends of the RNA isolated from HeLa cells transfected with the pSVA34 and pSVA29 series. Total cellular RNA was hybridized to an excess of *Hin*dIII/*Xho* I single-stranded probe (5′ end-labeled at the *Hin*dIII site; see Fig. 1*B*) and then analyzed. Lane G: G-sequence ladder (22) of the probe. MLP+1, nuclease S1-resistant fragments corresponding to specific transcription from the Ad2MLP. Arrowheads: see *Results*.

the results shown in Table 1 for pSVA34 and pSVA29, suggested that insertion of the 72-bp repeat close to the Ad2ML cap site can mask the effect of deletions of promoter elements. Since the effect of the 72-bp repeat decreases with increasing length

of intervening sequences (24), we inserted it in the pBR322 *Pvu* I site, about 4 kb upstream from the Ad2MLP (Figs. 1 and 2*A*) to minimize this masking effect. The amount of specific RNA in pSVPBA34- and pSVPBIA34-transfected cells was much lower than that in cells transfected with the corresponding pSVBA mutants (compare lanes 6 and 7 with lanes 2 and 3) and a difference could be seen between the recombinants with intact (pSVPBIA34; lane 5, Fig. 3*A*) and partially deleted (pSVPBIA29; lane 6, Fig. 3*A*) T-A-T-A box. It is striking that moving the 72-bp repeat in the *Pvu* I site resulted in a large increase in RNA initiated upstream from the deletion endpoint (arrowheads in Fig. 2*B*, compare pSVPBA34 and pSVPBIA34 with pSVA34, pSVBA34, and pSVBIA34). In fact nuclease S1 mapping (not shown) has shown that insertion of the 72-bp repeat in the *Pvu* I site promoted RNA synthesis from several nearby sites. This observation supports our previous hypothesis that the 72-bp repeat can act as an entry site for RNA polymerase B (24).

Nuclease S1 Mapping of RNA Synthesized *in Vivo* Shows that Sequences Located Upstream from the T-A-T-A Box Are Essential for Efficient Transcription from the Ad2MLP. RNA accumulated 48 hr after transfection of HeLa cells with the pSVPBIA recombinants was analyzed by quantitative nuclease S1 mapping (Fig. 3). Deletions up to position −97 had no effect on the amount of RNA initiated from the Ad2MLP (Fig. 3*A*, lanes 1–3). Deletions up to positions −62 and −34 resulted in decreases to 33% and 3%, respectively, of control values in the amount of specific RNA (Fig. 3*A*, lanes 3–5, and Table 2). Deletion of the first two bases (pSVPBIA29, lane 6) and of the entire T-A-T-A box (pSVPBIA21, lane 7) caused additional 40–50% reductions. It is remarkable that, even in the absence of the T-A-T-A box, the only band (excluding the deletion endpoint band) seen on the original autoradiogram corresponds to RNA initiated at the Ad2ML cap site. This band was absent in pEMPB*, which lacks all of the Ad2MLP region (Fig. 3*A*, lane 8). The differences found between the pSVPBIA recombinants in Fig. 3*A* were not artifactual since there was very little variation in RNA initiated upstream from the deletion endpoints (bands pointed out by arrowheads). In addition, a rabbit β-globin recombinant was cotransfected as an internal control with pSVPBIA97, -62, or -34. The intensity of the globin band (GLOB+1, Fig. 3*B*, lanes 1–3) was almost constant whereas the pattern of the pSVPBIA recombinants was very similar to that seen in Fig. 3*A*.

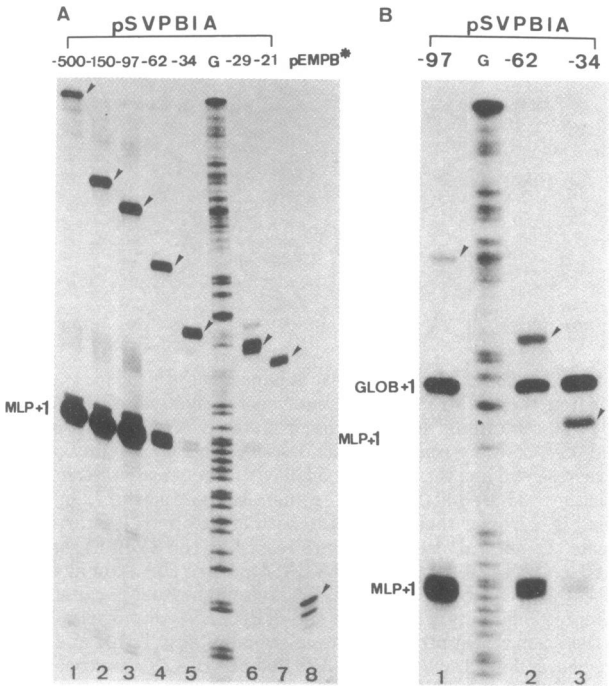

FIG. 3. Effect of deletions in the Ad2MLP region on specific *in vivo* transcription. (*A*) Nuclease S1 mapping (the *HindIII/Xho* I probe as in Fig. 2*B*) of Ad2MLP transcripts produced in HeLa cells transfected with pSVPBIA500, -150, -97, -62, -34, -29, and -21 (Fig. 1*A*). pEMPB* corresponds to pSVPBIA500 (Fig. 1*A*) but lacks the Ad2MLP sequence. (*B*) As in *A*, but the HeLa cells were cotransfected as indicated with pSVPBIA mutants and a polyoma–β-globin recombinant [pβ(244+)β in ref. 21]. The probe used to map the specific globin transcripts was as in ref. 21. GLOB+1, RNA initiated at the β-globin cap site. Experimental conditions and symbols are as in *Materials and Methods* and the legend to Fig. 2.

The Effect of the Upstream Sequences of the Ad2MLP Can Be Faithfully Reproduced *in Vitro*. Previous studies using an S100 extract and a run-off assay (26) did not reveal any effect of sequences upstream from the T-A-T-A box on Ad2MLP-specific *in vitro* transcription (1). The promoter sequence requirements were reinvestigated using the quantitative nuclease S1 assay while varying the type of cellular extract and the form of the template. As expected (1), deletions up to position −34 (Fig. 4*A*, lanes 1–3) did not affect specific initiation (MLP+1) from a linear template with the S100 extract whereas deletion of the T-A-T-A box abolished it (lane 4). In contrast, the effect of the upstream sequences was clearly seen with a whole cell extract (28) when either the nuclease S1 or the run-off assays was used (Fig. 4*B*). Deletions upstream from −62 and −34 resulted, respectively, in specific transcription decreases to 33% and 5%, respectively (lanes 1–3 and Table 2), of that of pSVPBIA97, which was as efficiently transcribed as the wild-type pSVPBIA500 (not shown). Similar results were obtained using the run-off assay (an Ad5E4 linear template was added as an internal control; Fig. 4*B*, lanes 5–7). No specific transcription was detected when the T-A-T-A box was deleted (lanes 4 and 8).

It has been suggested (18) that DNA free ends can serve as entry sites for RNA polymerase B and thereby mask the effect of promoter sequences with similar function. Therefore, circular DNAs (mainly superhelical; see Fig. 5 and *Discussion*) were used with either whole cell or S100 extracts (an Ad5E3 template was added as an internal control; see Fig. 5). With the whole cell extract, deletions upstream from −34 appeared to have a slightly more pronounced effect with circular than with linear templates (compare Fig. 5, lanes 4–6, with Fig. 4*B*, lanes 1–3). No specific transcripts were observed with the −21 mutant when circular templates were used (data not shown). To a lesser degree, an effect of the upstream sequences was also seen with the S100 extract and circular templates (compare lanes 1–3 with lanes 4–6), but it is striking that this effect was totally absent when linear templates were used (compare lanes 1–3 in Figs. 5 and 4*A*).

DISCUSSION

Our results show that sequences located upstream from the T-A-T-A box are important for efficient *in vivo* transcription from the Ad2MLP. Sequences upstream from −97 were not required, whereas sequences located between −97 and −34 played a major role. Our data (Fig. 3) suggest either that two sets of sequences are involved or that only one sequence, centered on position −62, is implicated. This second possibility is supported by a homology between the Ad2MLP sequence around −62 and the upstream homology sequence of the rabbit β-globin gene (3, 4), which was recently shown to be important for efficient transcription *in vivo* (ref. 31; P. Dierks, A. Van Ooyen, and C. Weissman, personal communication).

Table 2. Comparison of *in vivo* and *in vitro* efficiency of Ad2MLP mutants

Recombinant	*In vivo*	*In vitro* S100 extract Linear template	*In vitro* S100 extract Circular template	*In vitro* Whole cell extract Linear template	*In vitro* Whole cell extract Circular template
pSVPBIA97	100	100	100	100	100
pSVPBIA62	33	100	53	30	33
pSVPBIA34	3	80	20	5	3
pSVPBIA21	0.6	0	0	0	0

The intensity of the specific bands corresponding to initiation from the Ad2MLP (MLP+1; see Fig. 3*A*, lanes 3–5) was determined by densitometry of autoradiograms exposed for various periods of time. The values for pSVPBIA21 used as a circular template were taken from unpublished experiments. Results are expressed as percentage of the value obtained with pSVPBIA97.

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                                  −76
                                   |
Rabbit β-globin  5′− C-T-G-G-T-G-T-T-G-G-C-C-A-A-T − 3′
Ad2MLP           5′− T-A-G-G-T-G-T-A-G-G-C-C-A-C-G − 3′
                                   |
                                  −62
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Further deletion of the T-A-T-A box induced a further decrease in specific *in vivo* transcription. However, even when the T-A-T-A box was completely deleted, the residual transcription (0.6% of that of the wild-type) appeared to be still mainly initiated from the Ad2ML cap site, in contrast to the *in vitro* situation (Figs. 4 and 5 and ref. 1). Thus, some sequences located downstream from the T-A-T-A box could also be involved in the process that, *in vivo*, directs the transcription machinery to initiate at the cap site.

It is unlikely that insertion of the SV40 72-bp repeat more than 4 kb from the adenovirus sequence differentially modulates the activity of the different elements of the Ad2MLP, which appears to be stimulated as a whole. Indeed, the effect of the deletions was roughly the same after HeLa cell transfection with the pSVPBIA series as after microinjection in CV1 cell nuclei of recombinants lacking the 72-bp repeat. However, in-

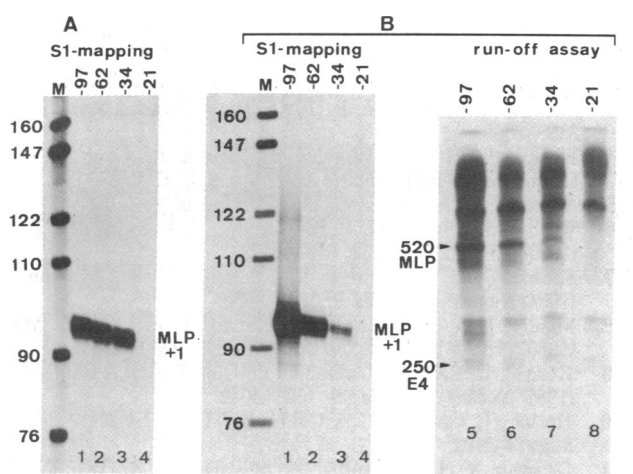

FIG. 4. Effect of deletions in the upstream region of the Ad2MLP on specific *in vitro* transcription of linear templates. (*A*) *In vitro* transcription with an S100 extract prepared from HeLa cells (26). Unlabeled RNA was synthesized in a three-fold standard reaction (27) in the presence of pSVPBIA97, -62, -34, and -21 (lanes 1–4) at 10 μg/ml, cut with *Taq* I (the *Taq* I fragment containing the Ad2MLP region extends from position 23 in pBR322 to position 4,739 in SV40). The *Hin*dIII/*Xho* I probe was used as in Fig. 2*B*. (*B*) *In vitro* transcription with a whole cell extract prepared as described (28). RNA was synthesized in a three-fold standard reaction (28) in the presence of the *Taq* I-digested templates (15 μg/ml) as in *A*. Lanes: 1–4, nuclease S1 mapping (as in *A*) of unlabeled RNA synthesized from pSVPBIA97, -62, -34, and -21; 5–8, electrophoretic analysis (5% acrylamide/8.3 M urea) of labeled run-off RNA synthesized under the same conditions but in the presence of [α-^{32}P]CTP. A *Sma* I/*Sph* I fragment (6 μg/ml) containing the adenovirus serotype 5 (Ad5) E4 promoter region was added as an internal control [the Ad5 *Sma* I fragment (98.4–100 map units; ref. 29) was cloned into pBR322]. The specific transcripts from the Ad2ML and Ad5E4 promoters are indicated by arrowheads (520 and 250 nucleotides long, respectively). The apparent difference in the position of the MLP+1 bands in lanes 1–3 (*A* vs. *B*) is due to electrophoretic distortion. Lane M: size markers ([^{32}P]-end-labeled *Msp* I fragments of pBR322).

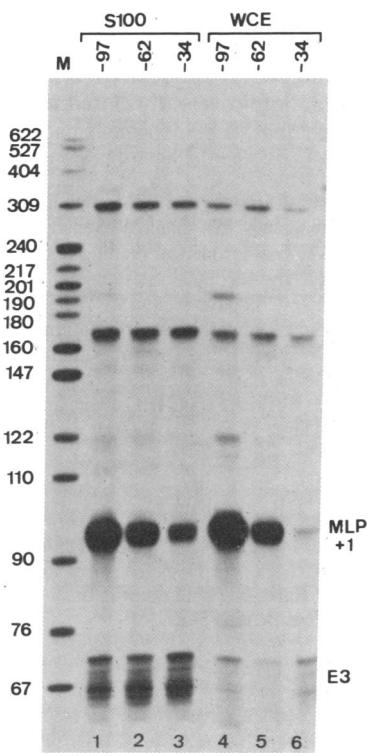

FIG. 5. Effect of deletions in the upstream region of the Ad2MLP on specific *in vitro* transcription of circular templates with an S100 extract or a whole cell extract (WCE). pSVPBIA97, -62, and -34 were transcribed as circular templates in the presence of S100 (lanes 1–3) or whole cell (lanes 4–6) extracts as described in Fig. 4. About 70% of the molecules were superhelical. A recombinant containing the Ad5 E3 promoter region [the Ad5 *Eco*RI C fragment (30) inserted into the *Eco*RI site of pBR322] was cotranscribed in each reaction (lanes 1–3, 3 μg/ml; 4–6, 5 μg/ml). The *Hin*dIII/*Xho* I probe was used as described in Fig. 2*B* and a 5′-end-labeled single-stranded *Sau*3A fragment spanning the E3 cap sites was used to map the E3 (RNA initiated at the Ad5E3 cap sites) transcripts. Lane M: size markers as in Fig. 4*B*.

sertion of the 72-bp repeat in a position too close to the Ad2MLP can mask the requirement for some of its elements (Fig. 2*B*). Obviously, an element with an effect similar to the 72-bp repeat is not present in the first 500 bp upstream from the Ad2ML cap site. Whether it is silent and made functional by a product(s) of other adenovirus genes or is present further upstream remains to be established.

The *in vivo* effect of the upstream sequences (−97 to −34) of the Ad2MLP can be faithfully reproduced *in vitro* with a whole cell extract and circular or linear templates (Figs. 3*A*, 4*B*, and 5 and Table 2). A similar observation was previously reported for a sea urchin histone *H2A* gene (18). As expected (1), no effect of the upstream sequences could be demonstrated with linear templates and an S100 extract. Assuming that DNA free ends could functionally replace the upstream sequences (18), this difference between the whole cell and the S100 extracts suggests the presence, in the whole cell extract, of protein(s) that bind to DNA ends and are absent in the S100 extract. However, this interpretation does not explain why, on circular templates (Fig. 5), the whole cell extract is more efficient than the S100 extract at mimicking the *in vivo* results. This difference cannot be attributed to the calf thymus RNA polymerase B that is added to the S100 extract, since the same results were obtained when transcription was carried out with S100 endogenous RNA polymerase B alone (results not shown). The whole cell extract may contain some factor(s) present in limiting amounts in the S100 extract that is required to reveal the effect of the upstream sequences. It is unlikely that such a factor(s) could be involved in the assembly of an "active" chromatin structure, since the superhelical DNA template was transformed within 5 min into covalently closed relaxed circles (results not shown) whereas, if nucleosomes had been formed, at least some superhelicity should have been conserved (32).

We are grateful to C. Kédinger and P. Moreau for many helpful discussions and gifts of materials. We thank W. Schaffner for his help with cell transfection and T. Leff for critical reading of the manuscript. We gratefully acknowledge the technical assistance of K. Dott, C. Hauss, B. Boulay, C. Kutschis, E. Badzinski, and C. Werlé. This investigation was supported by grants from the Centre National de la Recherche Scientifique (ATP 006520/50), the Institut National de la Santé et de la Recherche Médicale (CRT 80.1029), the Association pour le Développement de la Recherche sur le Cancer, and the Fondation Simone et Cino del Duca. R.H. is a Fellow of the Ligue Nationale Française contre le Cancer.

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