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Sequences controlling *in vitro* transcription of SV40 promoters

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A series of deletion mutants of SV40 were tested for early and late promoter activity *in vitro* in a transcription extract prepared from HeLa cells. These mutants had previously been characterized for expression *in vivo*. Transcription *in vitro* from both the SV40 early and late promoters was strongly dependent on an upstream region of DNA that contains six direct GC repeats. Sequences spanning two or more of these repeats stimulated transcription in a bidirectional fashion, at distances of 50-200 bp. These sequences may function by mediating the activity of a specific transcriptional factor. Little effect on transcription *in vitro* was observed upon deletion of the 72-bp enhancer elements. With this exception, the sequence dependence of early and late transcription *in vitro* was similar to that observed previously *in vivo*, both of the region including the GC repeats and of the early TATA sequence.

Key words: SV40/promoters/*in vitro* transcription

Introduction

To understand the molecular mechanisms by which eukaryotic RNA polymerase II initiates transcription, both the protein and DNA components of the reaction must be identified. Soluble cell-free extracts have been developed in which transcription by RNA polymerase II is accurately and specifically initiated (Weil *et al.*, 1979; Manley *et al.*, 1980). With such extracts, the DNA sequences required to potentiate transcription from a variety of promoters have been examined. Unfortunately, discrepancies remain between the DNA sequences required for *in vitro* transcription and those required *in vivo*. For most promoters, alterations only in the vicinity of the consensus TATA sequences, 20-30 bp upstream of initiation sites, have a pronounced effect on transcription *in vitro* (Corden *et al.*, 1980; Hu and Manley, 1981; Tsai *et al.*, 1981; Grosveld *et al.*, 1981). However, the critical sequences controlling the efficiency of transcription *in vivo* typically lie 50-150 bp upstream of initiation sites (Grosschedl and Birnstiel, 1980; Mellon *et al.*, 1981; Dierks *et al.*, 1981, 1983; Grosveld *et al.*, 1982; McKnight and Kingsbury, 1982; Pelham, 1982). In addition, enhancer elements, which can stimulate transcription *in vivo* over distances of several thousand base pairs (Gruss *et al.*, 1981; Banerji *et al.*, 1981; Moreau *et al.*, 1981; Fromm and Berg, 1983b), do not affect transcription *in vitro*. Recently, dependence on upstream sequences for transcription *in vitro* has been observed on some promoters, but in the range of a 2- to 6-fold effect (Tsuda and Suzuki, 1981; Grosschedl and Birnstiel, 1982; Hen *et al.*, 1982).

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The structure of the SV40 early and late promoters has been extensively studied *in vivo* (see Figure 5). The early promoter is comprised of the TATA sequence, which positions the major 5′ termini (Ghosh *et al.*, 1981; Benoist and Chambon, 1981); a sequence containing six direct GC repeats, 50-115 bp upstream of the major initiation site, which direct efficient trancription (Fromm and Berg, 1982, 1983a; Byrne *et al.*, 1983; Everett *et al.*, 1983), and the two 72-bp direct repeats, further upstream, which stimulate early transcription even when positioned large distances from the initiation site (Moreau *et al.*, 1981; Fromm and Berg, 1983b). The late promoter is more complex, consisting of many initiation sites within a 200-bp region of DNA (Ghosh *et al.*, 1978). The major late initiation site *in vivo* may be specified by a TATA-like sequence immediately upstream (Brady *et al.*, 1982), but no obvious TATA sequences are present for the other sites. The region containing six direct GC repeats, mentioned above, also plays a role in determining the efficiency of late transcription (Fromm and Berg, 1982). The requirements for other sequences are unclear.

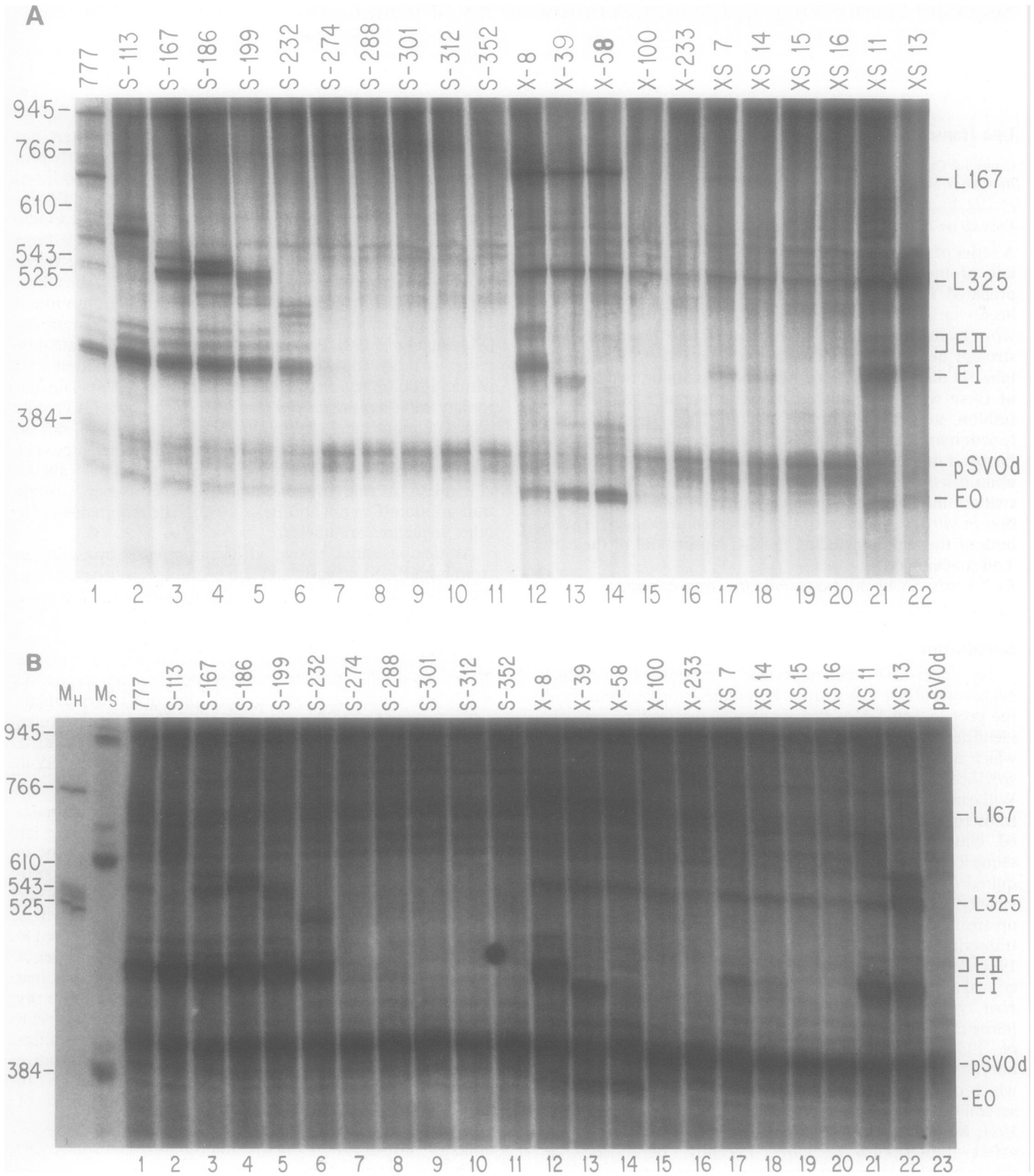
As demonstrated below, efficient *in vitro* transcription from both the SV40 early and late promoters is dependent on the same region of DNA, 50-200 bp upstream from the various initiation sites. This region contains six direct GC repeats, which comprise three direct 21-bp elements (two of which are precise repeats). The *in vitro* transcription data, like the *in vivo* results, indicate that these polar sequences contain redundant signals and act in both directions to stimulate transcription. In addition, transcription using a reconstituted system suggests that an SV40 promoter-specific and positive-acting factor is required for both early and late SV40 transcription *in vitro*.

Results

We have determined the DNA sequence required for *in vitro* transcription from both the early and late SV40 promoters. It was of major interest to investigate whether the same sequence dependences were evidenced *in vitro* in a whole cell soluble extract, as have been previously observed *in vivo*. For this comparison we have studied an extensive set of deletion mutants in the SV40 promoter region which had been previously characterized for *in vivo* expression by Fromm and Berg (1982, 1983a). This set of mutants is outlined in Figure 5. All the data to be presented concern transcription from linear DNA templates, but the same quantitative results have been obtained using circular DNA templates. In all cases the mutants were transcribed in whole cell extracts of HeLa cells.

Assay for identification and quantitation of RNA transcripts

To identify transcription from specific sites, an assay was developed using M13 single-stranded DNA to hybridize quantitatively radioactive RNA complementary to either the early (E) or late (L) strand of SV40. The RNA/DNA hybrids were bound to nitrocellulose filters by virtue of the single-stranded M13 DNA. Finally, the hybridized RNA was eluted from the filter and analyzed by gel electrophoresis. The utility of the technique is illustrated by comparing the results in


A
777
S-113
S-167
S-186
S-199
S-232
S-274
S-288
S-301
S-312
S-352
X-8
X-39
X-58
X-100
X-233
XS 7
XS 14
XS 15
XS 16
XS 11
XS 13
945
766
610
543
525
384
L167
L325
EII
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
B
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S-167
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XS 16
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pSVOd
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L167
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EII
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

Figure 1 with those in Figures 2 and 3. In Figure 1A the run-off transcripts were analyzed directly by gel electrophoresis. In Figures 2 and 3 the equivalent reactions were selected by hybridization to M13 recombinant DNA containing the SV40 E or L strand, respectively. The hybridization and filter-binding protocol eliminated non-specific background, permitting assignment of particular transcripts.

For quantitation, an additional template was added to all reactions as an internal control. The DNA of this template, pSV0d (Mellon *et al.*, 1981), contains the early SV40 promoter region (sequences 159−5171) and yields run-off transcripts of 407 and 411 bases. However, only 60 bases of this RNA are complementary to SV40 sequences in the M13 recombinant DNA and, under these hybridization conditions, the RNA was not quantitatively selected (compare Figures 1 and 2). For this reason, most quantitation was derived from scanning autoradiograms of polyacrylamide gels separating unhybridized reaction products.

Early SV40 promoter: characterization of transcription in absence of TATA sequence

SV40 early transcripts are initiated *in vitro* at adjacent sites clustered in three general regions (Figure 5; Mathis and Chambon, 1981; Hansen *et al.*, 1981; Ghosh and Lebowitz, 1981). The 5′ termini of the most prominent set of transcripts (EI) lie 28−33 bp downstream of a TATA sequence and correspond to *in vivo* transcripts synthesized prior to SV40 DNA replication. Transcripts are also initiated *in vitro* near the TATA sequence (EII) and probably correspond to *in vivo* transcripts synthesized following SV40 DNA replication. Finally, initiation also occurs *in vitro* at a site 114 bp downstream of the TATA sequence (E0); transcripts from this site have been observed *in vivo* only in mutants containing deletion of the TATA sequence (Benoist and Chambon, 1981). Run-off transcripts initiated at all three sites were readily observed in RNAs selected by hybridization to M13 recombinant DNAs complementary to the E strand (Figure 2, lane 1).

SV40 mutants with deletions of TATA sequences produce normal levels of early RNAs *in vivo*, with heterogeneous 5′ termini distributed over a region of 35 to 50 nucleotides (Ghosh *et al.*, 1981; Benoist and Chambon, 1981). Both the X-39 and X-58 mutants (Figure 5) display such a phenotype (Fromm and Berg, 1982). Transcription *in vitro* of these two mutants also yielded RNAs with heterogeneous 5′ termini (Figure 2, lanes 13, 14). In addition, the total efficiency of *in vitro* transcription of X-39 and X-58 DNAs was similar to that of the parental X-8 DNA (Figure 2, lanes 12−14; Table I). This was determined by summing the amount of transcription over all sites. Thus, in contrast to other promoters that have been studied *in vitro* and in agreement with previous studies on SV40, the TATA sequences of the early SV40 promoter seem only to influence the site of initiation and not the efficiency of transcription.

RNAs transcribed *in vitro* from X-39 and X-58 are initiated at similar positions to RNAs found in cells transformed by these two mutants (Fromm and Berg, 1982) (with the exception of the presence of the E0 start site *in vitro*). Among the heterogeneous sites used for initiation by each mutant lacking the TATA sequence, one site was transcribed at higher efficiency. For the 31-bp deletion mutant (X-39) and the 50-bp deletion mutant (X-58), the preferred sites were located 12 and 50 bp downstream of EI, respectively. Thus there is no strict correlation between the length of the deleted sequences and the most prominent site of initiation. The precise initiation sites seem to be determined by a combination of factors: preference for particular sequences at initiation sites and a general distance from some element upstream of the deleted sequences. In fact, on close examination of transcription from wild-type templates, weak *in vitro* transcription was observed from initiation sites prominently used in TATA-deleted mutants (U. Hansen, unpublished observations and Figure 2). The preferred site used in transcribing X-39 has also been detected *in vivo* in wild-type transcription (Ghosh *et al.*, 1981). The level of mutant transcription from the E0 site concurs with this hypothesis. Initiation at E0 increased in frequency in both mutants, becoming more prominent with the larger deletion (see also Mathis and Chambon, 1981).

SV40 early promoter: dependence on DNA sequences containing the GC repeats

In vivo, the efficiency of early transcription is dependent on two sets of sequences: (i) the 72-bp enhancer elements and (ii) sequences containing the six direct repeats of the sequence PyPyCCGCCC. The six direct GC repeats also form three 21-bp elements, two of which are precise repeats. In summary of all their *in vivo* results, Fromm and Berg (1982) suggested that the amount of early transcription correlated with the number of GC repeats. We have examined the *in vitro* transcriptional properties of three sets of the Fromm and Berg mutants.

The first set of deletion mutants, members of the S-series, have a common end point at the *Hpa*II site within the late SV40 leader region and are missing increasing lengths of sequences towards the early initiation sites (Fromm and Berg, 1982). The mutants of interest for analyzing dependence of early transcription on the region containing the GC repeats are: S-232, S-274, S-288, and S-301 and S-312 (see Figure 5). It is clear that early transcriptional activity markedly decreased (13- and 25-fold) upon deletion of sequences to nucleotides 72 and 58, which include three and four GC repeats, respectively (Figure 2, lanes 6−8). With removal of all (S-312) or almost all (S-301) of the region containing GC repeats (lanes 9, 10), no initiation of early transcription was observed at

Fig. 1. Transcription of mutant SV40 DNA templates, at high and low DNA concentrations. Transcriptions using HeLa whole cell extract were performed as described in Materials and methods. One third of each reaction was analyzed directly by electrophoresis on a thin denaturing polyacrylamide gel (see Materials and methods). **(A)** The experimental template DNA, digested with *Sau*3A, is indicated at the top of each lane and was present at 30 μg/ml, except for 777 DNA, which was present at 21 μg/ml. All transcriptions were also performed with 7.5 μg/ml of the pSV0d template, digested with *Bam*HI, as an internal control. 15 μCi of [α-^{32}P]UTP, at 350 Ci/mmol, was present in each total reaction. The marker lengths indicated to the left of the lanes were obtained by electrophoresis of samples of the same markers described in **(B)**. The labels on the right side of the gel indicate the initiation sites of the various RNA species in the gel. **(B)** The experimental template DNA in each reaction is indicated at the top of each lane, and was present at 4.0 μg/ml, except for 777 DNA, which was present at 2.8 μg/ml. All transcriptions were performed in the presence of 18 μg/ml poly[d(I-C)].[d(I-C)] (Hansen *et al.*, 1981), as well as 2.5 μg/ml of the pSV0d template. Lane 23 contained only the control DNA. Note that transcription of pSV0d produced RNAs equivalent to both EI (labeled pSV0d) and EII (immediately above). No pSV0d EO transcript is produced because that initiation site is missing in the plasmid. 20 μCi of [α-^{32}P]UTP, at 220 Ci/mmol, was present in each total reaction. M_H and M_S contain denatured fragments of SV40 DNA, digested with *Hinf*I and *Sau*3A, respectively, and then labeled with [α-^{32}P]dNTPs using the large fragment of *E. coli* DNA polymerase I. The lengths of the DNA fragments are indicated at the left.

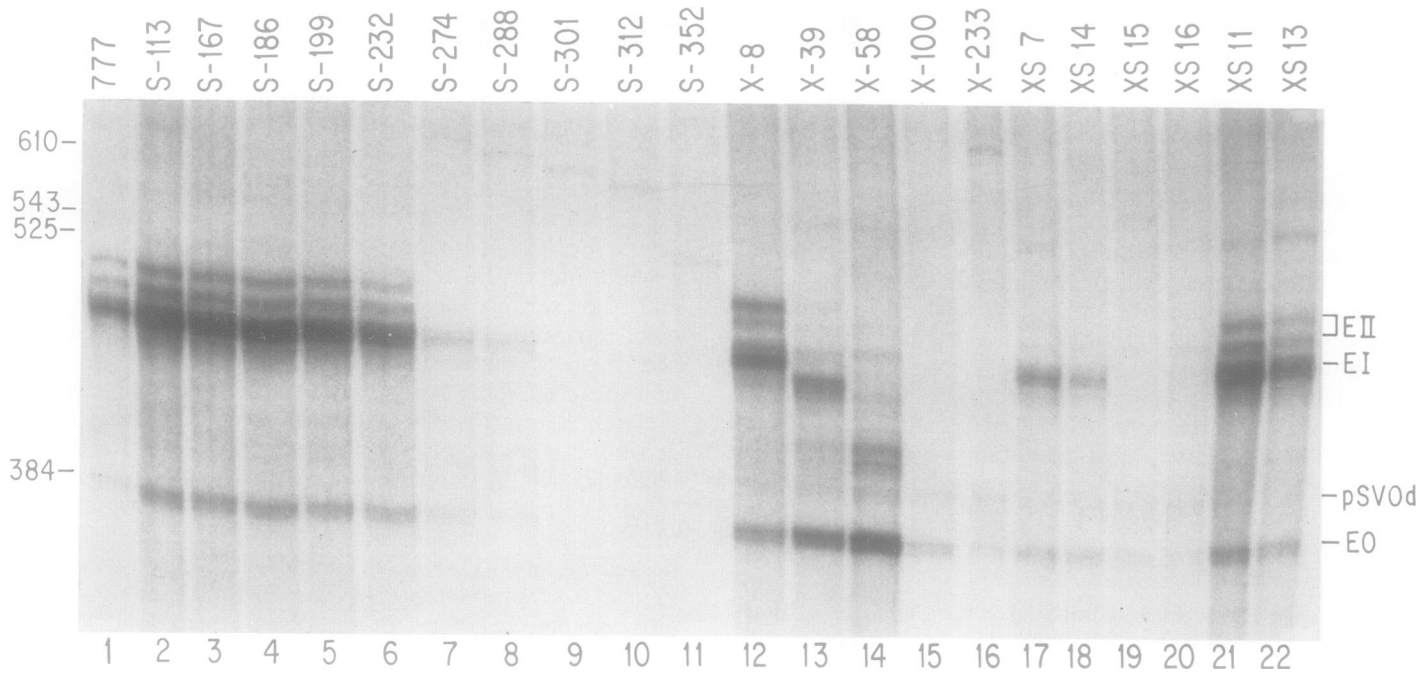


Fig. 2. Early transcripts of mutant SV40 DNA templates. Early RNA from samples identical to those in Figure 1A were selected by hybridization to 24 μg/ml mSV03 DNA (an M13-SV40 recombinant containing the E strand of the 1350-bp *Sau*3A fragment of SV40, nucleotides 4770–877, in M13mp6; Hay and De Pamphilis, 1981), and filtration onto nitrocellulose filters (see Materials and methods). Following elution from the filter, the RNA was sized by electrophoresis on a thin denaturing polyacrylamide gel. The length markers were as described in Figure 1.

EI. Only a basal level of initiation at E0 persisted in the absence of these sequences (see Discussion). Since both mutants S-301 and S-312 retain the TATA sequence, this element is not sufficient for transcription from EI.

The second set of mutants examined, members of the XS-series, have a common end point at nucleotide 108 and are missing increasing lengths of sequences toward the early initiation sites (Fromm and Berg, 1982). The mutants XS7 XS14 and XS15 were derived from S-274, S-288 and S-301, respectively. Mutant XS16 contains a deletion of the entire region containing the GC repeats and of some of the AT-rich sequences, but retains the TATA sequence. As before, deletion of sequences to nucleotides 72 and 58 (removing three and four GC repeats, respectively) decreased early transcription significantly (7- and 15-fold), and deletion of sequences to nucleotides 45 and 25 dropped the efficiency of transcription to a virtually undetectable level (Figure 2, lanes 17–20).

Finally members of the X-series mutants have a common end point just upstream of EI and are missing increasing lengths towards the late initiation sites (Fromm and Berg, 1982). Mutants X-39 and X-58, which transcribed efficiently in the absence of the TATA sequence (see above), retain sequences to nucleotides 34 and 53, spanning six and five GC repeats, respectively (lanes 13, 14). Mutant X-233 (with deletion of the complete GC region as well as most of the 72-bp repeats) did not support early transcription outside of the basal transcription from E0 (Figure 2, lane 16). Mutant X-100, retaining sequences to nucleotide 95 and including a single GC repeat, also gave no detectable transcription above the basal E0 level *in vitro* (Figure 2, lane 15). *In vivo* early transcription was also not detected in this mutant (Fromm and Berg, 1982).

Late SV40 promoter: dependence on DNA sequences containing the GC repeats

The 5′ termini of the major *in vitro* SV40 late transcripts can be assigned to the following locations: prominent RNAs initiating at nucleotides 325, 264 and 167, and minor RNAs initiating at nucleotides 290, 239, 192 and 140 (see Figure 5). These assignments were based on several types of data: (i) an analysis of 5′ termini of both *in vitro* and *in vivo* RNA by hybridization with short end-labeled DNA probes and digestion with either S1 or mung bean nuclease (U. Hansen, unpublished data); (ii) determination of lengths of short run-off transcripts on denaturing polyacrylamide gels (U. Hansen, unpublished data and Figure 3); and (iii) published 5′ mapping and cap sequence analysis data (Ghosh *et al.*, 1978; Haegeman and Fiers, 1978; Contreras and Fiers, 1981). Although the major initiation site *in vivo* in wild-type-infected cells is at nucleotide 325, all the other initiation sites have also been detected *in vivo*. In some viral deletion mutants, these normally minor initiation sites can actually be very efficiently utilized (for example, Ghosh *et al.*, 1982). Transcripts initiated *in vitro* at all seven sites can be seen in run-off RNAs from either viral 777 or X-8 DNA templates (see Figure 3, lanes 1 and 12, respectively). All of these transcripts are sensitive to 1 μg/ml α-amanitin (data not shown).

Fromm and Berg (1982) have shown that SV40 late gene expression *in vivo* is also dependent on DNA sequences in the GC repeat region. These sequences lie over 200 bp upstream of the major late *in vivo* initiation site (nucleotide 325). Again, the three sets of mutants (X-series, XS-series and S-series) were tested for initiation of trancription *in vitro*

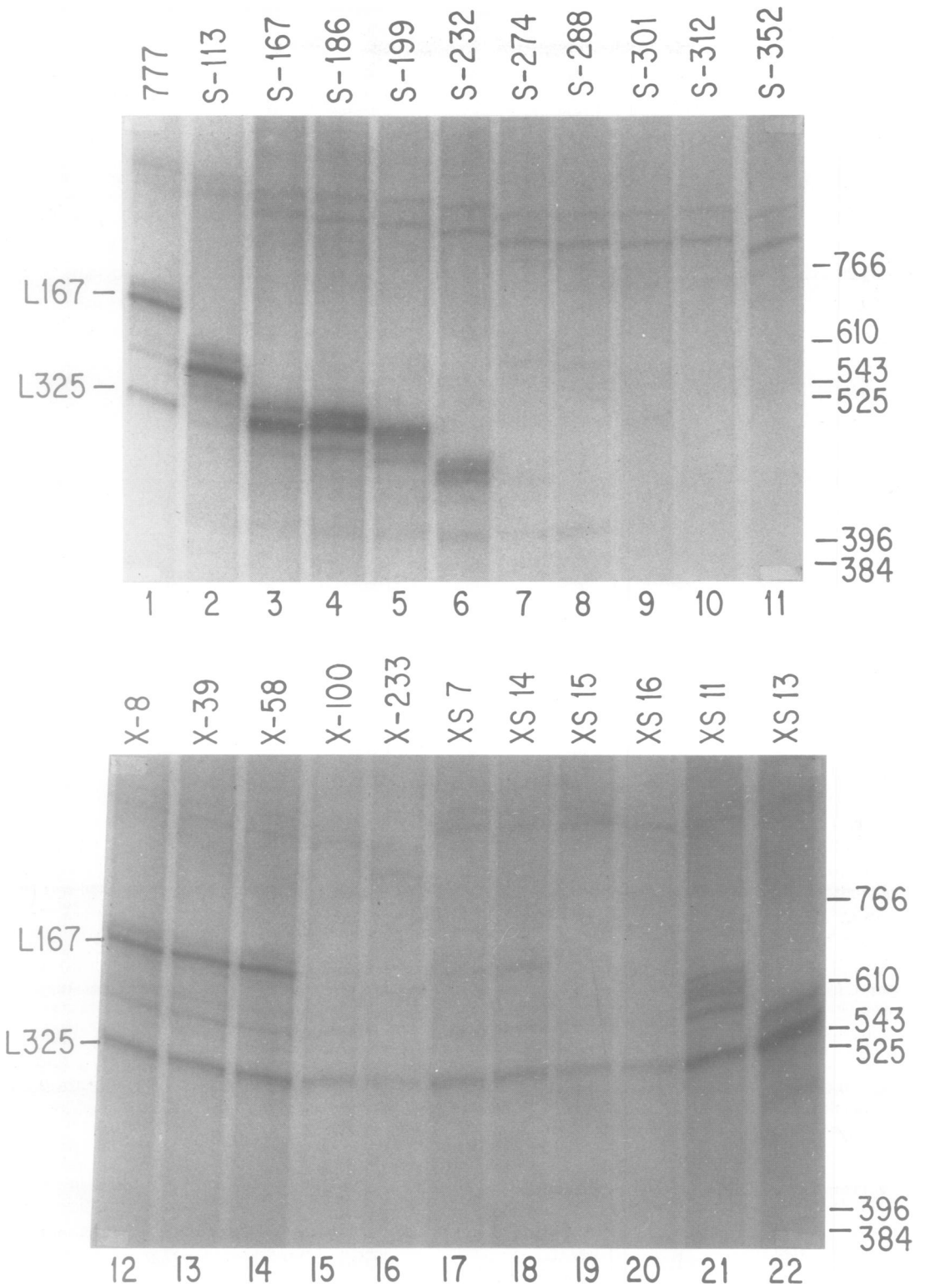


Fig. 3. Late transcripts of mutant SV40 DNA templates. Late RNA from samples identical to those in Figure 1A were selected by hybridization to 21 μg/ml mSV04 single-stranded viral DNA (an M13-SV40 recombinant containing the L strand of the 1350-bp *Sau*3A fragment of SV40 in M13mp6, Hay and De Pamphilis, 1981) and filtration onto nitrocellulose filters. Following elution from the filters, the RNA was sized by electrophoresis on a thick denaturing polyacrylamide gel. The length markers were as described in Figure 1. Whereas the wild-type lengths of transcripts **(lane 1)** were observed for many of the mutants **(lanes 12–20, 22)**, novel lengths, sometimes due to transcription from novel initiation sites, were evident for others **(lanes 2–6, 21)**. Transcription from S-113 and S-167 **(lanes 2** and **3**, respectively), was initiated with approximate wild-type frequencies at the remaining normal sites: nucleotides 167 and 140. Transcription from S-186 **(lane 4)**, which deletes the site at 167, resulted in enhanced transcription from nucleotide 140 and novel *in vitro* transcription from nucleotide 120 (transcription at this site is also seen *in vivo*). Transcription from S-199 **(lane 5)**, which deletes to within 7 bp of nucleotide 140, retained some initiation from nucleotide 140, with enhanced transcription from nucleotide 120. In addition, initiation of trancription was observed from a site within the late leader region (around nucleotide 360). Finally, transcription from S-232 **(lane 6)**, which deletes all upstream initiation sites, proceeded efficiently from new sites within the late leader (around nucleotides 360, 370, 380). For mutant XS 11 **(lane 21)**, three new initiation sites were utilized between nucleotides 239 and 197 (its deletion end point).

Table I. Comparison of *in vitro* and *in vivo* data concerning transcription of the SV40 early and late promoters

	Early transcription							Late transcription					
	In vitro				*In vivo*			*In vitro*					*In vivo*
	(% of wild-type)				(% of wild-type)			(% of wild-type)					
Mutant	EII	EI	E0	E (total)	RNA (β-globin)	Transformation	E +	L167	L264	L325	L(major)	L(total)	L +
S-113	100(100)	100(100)	100(100)		100	100					55(120)	31(80)	
S-167	100(100)	100(110)	120(92)			60	+				74(210)	46(97)	+
S-186	110(87)	110(80)	130(83)			50	+/−				82(190)	61(130)	+
S-199	81(97)	75(88)	120(83)		1	50	+/−				30(100)	23(73)	+
S-232	39(77)	55(59)	80(78)		<1	40	−	N.A.	N.A.	N.A.	25(150)	16(69)	+
S-274		7.7(6.0)	26(39)		<1	30	−				<1.7(<25)	<1(<12)	−
S-288		5.5(3.9)	36(36)			20	−					<1	−
S-301		<0.4(<0.5)	24(39)			15	−					<1	-
S-312		<0.4(<0.5)	19(36)		<1	2	−					<1	−
S-352		<0.4(<0.5)	21(31)		<1	0	−					<1	−
X-8	120(69)	72(74)	160(100)	100(100)	80	100		100(100)	100(100)	100(100)	100(100)		
X-39	N.A.	30*(41*)	300(210)	73(88)	70	40		82(66)	85(100)	100(100)			
X-58	N.A.	21*(25*)	460(310)	70(74)	80	40		87(83)	95(140)	100(100)			
X-100	N.A.	N.A.	47(53)	8.2	3	<2		4.5(<4)	25(40)	37(45)			
X-233	N.A.	N.A.	34(53)	5.0	3	<1		N.A.	<6.6	18(25)			
XS7	17	14(9.4)	42(47)		80	75	+	12	29	54(61)			+
XS14	4.9	10(6.8)	42(78)		115	50	+	17	45	64(71)			+
XS15	2.0	1.1	29(42)		7	10	+/−	1.9	22	44(59)			+/−
XS16	2.8	0.8	22(47)			10	−	<1.2	20	35(38)			−
XS11	92(72)	88(75)	180(110)			100	+	N.A.	270(260)	160(86)		110(110)	+
XS13	92(49)	88(56)	180(53)			40	−	N.A.	N.A.	590(480)		190(290)	+

The *in vitro* transcription data in Figures 1–3 and from similar data obtained at low SV40 DNA concentrations were quantitated by scanning the autoradiograms with a Joyce-Loebl double-beam recording microdensitometer and determining peak areas by means of a Hewlett Packard 9864A digitizer interfaced with a 9815A calculator. The relative intensities of the bands of RNA are represented as the percentage of 'wild-type' transcription from the various initiation sites with the 'wild-type' internal controls being X-8 for late transcription and S-113 for early transcription. The relative intensities of initiation at the various sites at high DNA concentration (or low DNA concentration) are as follows: EII, 0.28 (0.16); EI, 1.00 (1.00); E0, 0.11 (0.094); L167, 1.23 (0.14); L264, 0.28 (0.027); L325, 0.68 (0.13). It is clear that EI was by far the most prominently used initiation site at low DNA concentrations, but was matched in activity by late transcription at high DNA concentrations (see also Rio *et al.*, 1980). Two sets of numbers are given for the *in vitro* data. The one in parenthesis is that obtained at low SV40 DNA concentration, the other is that obtained at high SV40 DNA concentration. In cases where novel initiation sites are used in the mutants, total transcription, as well as transcription from the prominently used sites [L(major), EI*] were measured. The wild-type L(major) was taken to be L167 from X-8. N.A. indicates that it is not applicable, generally because the initiation sites have been deleted. The *in vivo* data were reported previously by Fromm and Berg (1982, 1983b), and are shown here for comparison. E^+ and L^+ indicate the ability of the mutant SV40 DNAs, when excised from the pBR322 sequences, to complement early (tsA58) and late (tsB201) SV40 mutants, respectively (Fromm and Berg, 1982). The ability of the mutants to transform was determined by transfecting the plasmid DNAs into rat F-111 cells and determining the number of foci obtained in 3–4 weeks, relative to a wild-type plasmid (Fromm and Berg, 1982). *In vivo* RNA levels were determined by fusing a β-globin coding region to the wild-type and mutant SV40 promoters, transfecting these DNAs into CV1 cells, and measuring the amount of β-globin RNA in the cells 48 h later (Fromm and Berg, 1983a). In addition Fromm and Berg (1982) have measured the amount of late RNA produced *in vivo* by the mutant S-232 to be ~25% that of wild-type.

from the various late sites discussed above. In the X-series, mutants X-8 (with a *Xho*I linker substituted in the origin of replication), X-39 (deletion of AT-rich sequences) and X-58 (deletion of AT-rich sequences and one GC repeat) were transcribed to wild-type patterns of late RNA (Figure 3, lanes 12–14). Substantial reductions in the efficiency of initiation from all sites were observed in X-100, however, in which sequences are retained to nucleotide 95, including only a single GC repeat (lane 15). Closer examination of the X-100 results showed that initiation at nucleotide 167 was more drastically reduced than initiation around nucleotide 325 (Table I). Finally, mutant X-233 (with deletion of the entire GC repeat region and most of the 72-bp repeats) gave an even further reduction of transcription from the remaining initiation sites (nucleotides 325, 290 and 264) (lane 16).

Similar results were obtained from transcription of the XS-mutants, which contain a common deletion end point at nucleotide 108. This end point is 59 and 217 bp upstream of the initiation sites at 167 and 325, respectively. XS7 and XS14 (deletions to nucleotides 72 and 58, leaving three and two GC repeats, respectively) gave reduced levels of transcription from all initiation sites, but affected initiation at nucleotide 167 to a greater extent than that at nucleotide 325 (Figure 3, lanes 17 and 18; Table I). XS15 and XS16 (deletion to nucleotides 45 and 25, leaving potentially a single and no GC repeats, respectively) gave virtually undetectable transcription

from position 167, and lower levels of transcription from nucleotides 239 through 325 (lanes 19 and 20).

The situation was similar but more complex when the S-series mutants were transcribed. These mutants contain a common end point at nucleotide 346, within the late transcripts, and are missing increasing lengths of sequences toward the GC repeat region. Both the sites of initiation and efficiencies of transcription vary between mutants (see legend to Figure 3). In general, however, the total level of late transcription from mutants S-113, S-167, S-186, S-199 and S-232 was similar to that from wild-type DNA (Figure 3, lanes 1–6). Mutants S-274, S-288, S-301, S-312 and S-352, which delete half or more of the region containing the GC repeats, gave no detectable late transcripts (lanes 7–11). This is not merely due to the deletion of all available initiation sites, because mutant S-232, which retains no wild-type initiation sites, is transcribed from sites in the late leader (legend to Figure 3) that are present in all the remaining mutants. These results, with those of the XS-series, suggest that late transcription from the 167 site is totally dependent on sequences containing the GC repeats, while late transcription from the 264 and 325 sites are strongly dependent on these sequences.

Deletions that place the GC repeat region near the downstream late initiation sites gave higher levels of transcription from these sites. Mutant XS11 (lacking one 72-bp repeat) gave enhanced transcription from site 264, but normal levels of transcription from sites 290 and 325 (lane 21), while mutant XS13 (lacking both 72-bp repeats) gave 5-fold enhanced transcription from those same two remaining sites (lane 22).

Requirement of specific factor for SV40 transcription

The novel DNA sequence dependence for *in vitro* transcription from the SV40 early and late promoters suggested a novel mechanism of initiation, and perhaps requirements for novel initiation factors. It has been demonstrated that specific initiation of transcription from the major late promoter (MLP) of adenovirus can be reconstituted with three factors and RNA polymerase II (Samuels *et al.*, 1982). The efficiency of transcription from the MLP is, however, strongly dependent on the TATA sequence. Thus, the activities of early and late SV40 promoters were compared with those of the MLP in both the whole cell extract and the reconstituted system. SV40 and MLP templates were co-transcribed and analyzed by hybridization with an excess of a mixture of M13 DNAs. The hybrids were digested with T1 nuclease and selected by filtration onto nitrocellulose filters. In all cases, the M13 insert included sequences encoding the 5′ termini of the RNA; therefore, the length of the protected RNA could be used to position the initiation sites. The M13 DNAs, described in the legend to Figure 4, protect RNAs of 197 bases from the MLP, 495–511 bases from EII, 468–473 bases from EI, and 386 bases from E0. RNAs of 558, 619 and 716 bases are protected from L325, L264 and L167, respectively.

Comparison of the early SV40 and MLP promoters in the reconstituted and whole cell extract systems revealed that the relative efficiency of the SV40 (EI) promoter decreased by ~60-fold in the fractionated system (Figure 4A, compare lanes 1 and 2). The low activity of the EI promoter did not change over a 10-fold range of DNA concentrations (lanes 1–6, and A. Fire and U. Hansen, unpublished observations). Transcription of E0 and EII was retained in the reconstituted system at a low basal level. By addition of a small amount of whole cell extract (1/9 the amount usually used), a partial restoration of early SV40 transcription was observed (lanes 9–11). Heating the whole cell extract aliquot at 65°C for 5 min prior to its addition abolished the enhancement (lane 12).

When the relative efficiencies of the late SV40 and MLP were compared in the whole cell extract and reconstituted systems, transcription from the SV40 promoter sites was inefficient in the latter system (Figure 4B, lanes 1–6). The reduction in activity was quite dramatic for the upstream late initiation site at nucleotide 167 (10-fold, at the minimum) but less dramatic for the downstream initiation sites at nucleotides 264 and 325 (3- to 8-fold). One again, addition of a small amount of whole cell extract restored transcription from the late initiation sites (Figure 4B, lanes 9–12). Restoration of transcription was most dramatic for initiation at nucleotide 167.

Since the reconstituted system has been shown to transcribe several promoters (the adenovirus MLP, EIV and EIA promoters; Samuels *et al.*, 1982), these data suggest that a specific factor is necessary for *in vitro* activity of SV40 promoters. As this work was in progress, Dynan and Tjian (1983) published an elegant study of this same SV40-specific factor. They fractionated it away from the other transcriptional factors in the extract and identified two additional promoters (human β-globin and the long terminal repeat of avian sarcoma virus) on which it had no demonstrable effect.

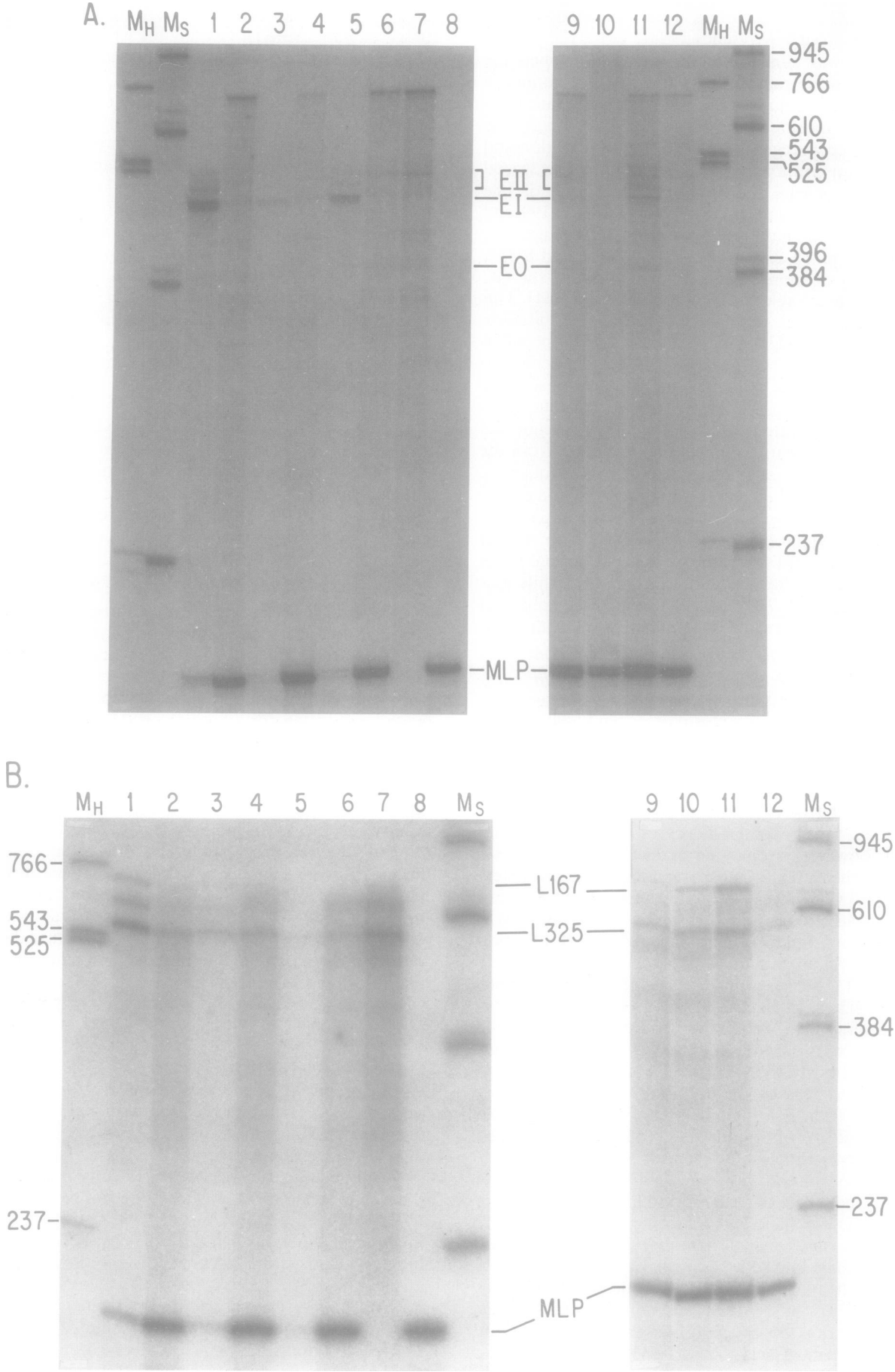
Competition between SV40 promoters in vitro

Unlike most promoters, *in vitro* transcription of SV40 in a whole cell extract decreases specifically with addition of higher concentrations of SV40 DNA (and at lower concentrations than those which generally inhibit transcription). The specificity of this effect for the addition of SV40 DNA suggests competition for a limiting specific factor. A similar effect can be seen in Figure 1A where different mutant SV40 templates affected the transcription of the internal control, pSV0d. Whenever the mutant template supported early and late transcription, i.e., whenever the mutant template contained the region including the GC repeats, transcription from pSV0d was depressed. This effect was only observed when high concentations of SV40 DNA were present (compare Figure 1A and B) though, at low SV40 DNA concentrations, the total DNA concentration was held constant by the addition of carrier DNA. The data suggest that the competition is mediated by the limiting specific factor binding to the region of the GC repeats.

Discussion

The SV40 early and late promoters are quite unique in their *in vitro* properties. Previous experiments concerning the SV40 early promoter had indicated that removal of the TATA sequences resulted in increased heterogeneity in initiation sites (Mathis and Chambon, 1981). Removal of sequences 70–140 bp upstream of the major early initiation site (EI) drastically reduced the efficiency of transcription (Myers *et al.*, 1981; Lebowitz and Ghosh, 1982). In the latter studies, however, the mutants had not been characterized *in vivo*. We have chracterized the *in vitro* transcription of both the early and late SV40 promoters, using an extensive set of deletion mutants previously characterized *in vivo*.

The TATA element of the early promoter is unique relative to similar regions in other promoters: (i) mutants containing the TATA sequences but lacking the upstream repeats do not


A.
MH MS 1 2 3 4 5 6 7 8
9 10 11 12 MH MS
945
766
610
543
525
EII
EI
EO
396
384
237
MLP
B.
MH 1 2 3 4 5 6 7 8 MS
9 10 11 12 MS
766
543
525
237
L167
L325
MLP
945
610
384
237

direct transcription *in vitro* (also see Myers *et al.*, 1981), and (ii) mutants lacking the TATA sequences but retaining the upstream repeats produce heterogeneously initiated transcripts, but with efficiency similar to wild-type templates. Thus, the sole function of the TATA sequences of the early SV40 promoter is apparently to position RNA polymerase II to initiate transcription at EI. This property of the SV40 TATA element might be related to the 5′-flanking very AT-rich sequence. Most TATA sequences are embedded in GC-rich segments.

The DNA sequence requirements for efficient transcription *in vitro* also closely resemble the *in vivo* results, with the exception of the effect of the 72-bp enhancer element on the early promoter (Table I). Transcription from both the early and late SV40 promoters is strongly dependent on sequences spanning the set of six PyPyCCGCCC repeats positioned 55 or more base pairs upstream from the various initiation sites. The amount of *in vitro* transcription is roughly proportional to the amount of DNA encompassing this region. The dependence of SV40 transcription *in vitro* on the GC repeat region is dramatic (at least 50- to 100-fold) for the immediately flanking sites (L167 and EI). Those distal sites which retain a low basal level of transcription in the absence of this region depend on additional and independent DNA sequences *in vitro* (nucleotides 294−304 for L325, Brady *et al.*, 1982; nucleotides contained in 5140−5238 for E0, U. Hansen, unpublished observations). Although DNA sequences sufficient for initiation at these distal sites need not include the region of the GC repeats, the region still significantly enhances such transcription (3- to 5-fold), over distances of 222 and 135 bp for L325 and E0, respectively.

The region of the GC repeats contains two obvious sequence elements: the 6-fold direct repeats (PyPyCCGCCC) and the partial 3-fold 21-bp direct repeat (TCCCGCCCCTA-ACTCCGCCCA). The latter repeats directly abut one another. Mutants in this region with partial deletions from either direction retain partial transcriptional activity, suggesting that the region contains an overlapping or redundant set of signals. A deletion mutant containing only one 21-bp repeat (and thus two GC repeats) supported a 5−10% level of early *in vitro* transcription (S-288, XS14; see Table I). (It should be noted that deletion mutants which contain two or three GC repeats, XS14 and XS7, show greater early transcription activity *in vivo* than *in vitro*. This perhaps is due to an enhancing effect *in vivo* of the 72-bp region.) *In vivo*, deletion mutants with only one GC repeat retain 7−25% transcriptional activity (see XS2, XS15, Fromm and Berg, 1983a). Thus, as has been previously suggested, the GC repeat might be the critical element in stimulation of both early and late transcription.

There are two striking features of the stimulation of transcription by the GC repeat region: (i) initiation is stimulated in a bidirectional fashion, and (ii) the stimulation is more pronounced at the nearest initiation sites (or over the shortest distances). The precise positions of these initiation sites are not determined by the GC repeat region, but rather by local DNA sequences, sometimes including a TATA sequence. These features suggest that (both *in vitro* and *in vivo*) the process mediated by the GC repeat region enhances polymerase II recognition of initiation sites in flanking sequences. That the GC repeat region can stimulate early transcription in a non-polar fashion has been demonstrated *in vivo* by inversion at its normal location (Everett *et al.*, 1983).

The final unique feature of *in vitro* transcription from the SV40 promoters is the requirement for a specific factor (Figure 5 and Dynan and Tjian, 1983). Transcription of the major late promoter of adenovirus can be reconstituted with three fractions of a cellular extract and purified RNA polymerase II (Samuels *et al.*, 1982). These components could not, however, reconstitute significant transcription from the SV40 promoters. Such transcription could only be restored with the addition of a small amount of whole cell extract. This suggests that a specific, positive-acting factor is required for SV40 *in vitro* transcription. Furthermore, the degree to which the SV40 initiation sites are dependent on the factor is paralleled by their degree of dependence on the region containing the GC repeats. Initiation sites strongly dependent on the factor are also strongly dependent on the repeats (EI, L167). Thus, it is possible that the factor activates transcription through the region of the GC repeats. Additional evidence relating the activity of the SV40-specific factor with the GC repeat region was obtained from DNA competition experiments. High concentrations of SV40 DNA specifically inhibit transcription from the SV40 initiation sites. The ability to compete in this reaction is dependent on the presence of the GC repeat region (Figure 1 and U. Hansen, unpublished observations).

There are indications that the SV40-specific transcriptional factor may actually be a more general transcriptional factor. To begin with, it is a cellular factor, found in uninfected cellular extracts. But more suggestively, the SV40 sequences which mediate its function have analogs in several other promoters. *In vivo* transcription of the herpes thymidine kinase gene has been shown to be dramatically dependent on two upstream sequences, one being CCCCGCCC and the other being GGGCGGCG (McKnight and Kingsbury, 1982). These

Fig. 4. Transcription of early and late SV40 promoters in a fractionated extract. Transcription from the SV40 promoters was compared with that from the adenovirus major late promoter (MLP) by transcribing both templates simultaneously either in whole cell extract (**lanes 1, 3, 5**), or in a fractionated system (**lanes 2, 4, 6−8**), or in the fractionated system supplemented with small amounts of whole cell extract (**lanes 9−12**). Whereas 9 μl whole cell extract were normally used in the transcription reaction (**lanes 1, 3, 5**), only 0.25 μl, 0.5 μl and 1.0 μl were added to supplement reactions for **lanes 9, 10** and **11**, respectively. The reaction for **lane 12** received 1.0 μl whole cell extract which had been previously heated to 65°C for 5 min. All transcriptions were performed using the preincubation/pulse/chase protocol described in Materials and methods. The reactions containing only whole cell extract (**lanes 1, 3, 5**) were supplemented with 18 μg/ml poly[d(A-T)].[d(A-T)] as carrier DNA in the preincubation. The SV40 template was viral DNA digested with *Taq*I endonuclease; the MLP template was pFLBH DNA (Samuels *et al.*, 1982) digested with *Pst*I endonuclease. The markers M_H and M_S are as described in Figure 1. (**A**) The transcription reactions contained the following DNA concentrations in the preincubation reactions: 8 μg/ml SV40 DNA, 2 μg/ml MLP DNA (**lanes 1, 2**); 2 μg/ml SV40 DNA, 2 μg/ml MLP DNA (**lanes 3, 4**); 4 μg/ml SV40 DNA, 2 μg/ml MLP DNA (**lanes 5, 6, 9−12**); 4 μg/ml SV40 DNA (**lane 7**); and 4 μg/ml MLP DNA (**lane 8**). Two-ninths of each reaction was hybridized to a mixture of 20 μg/ml DNAs each of mSV03 (see legend to Figure 2) and M13 XH11 [an M13-adenovirus recombinant containing a 450 base insert including the MLP and bound by *Xho*I and *Hind*III restriction sites, in M13mp11 (M .Samuels, A. Fire, and P. Sharp, in preparation)]. The hybrids were digested with T1 nuclease and filtered (see Materials and methods). The eluted RNA was analyzed on a thin denaturing polyacrylamide gel. (**B**) The transcription reactions were as described in (**A**) with the exception that the reactions for **lanes 3** and **4** contained 4 μg/ml SV40 DNA, 2 μg/ml MLP DNA and the reactions for **lanes 5** and **6** contained 2 μg/ml SV40 DNA, 2 μg/ml MLP DNA in the preincubations. Two-ninths of each reaction was hybridized to a mixture of 20 μg/ml DNAs each of mSV04 (see legend to Figure 3) and M13 XH11 [see (**A**)]. The hybrids were digested with T1 nuclease and filtered. The eluted RNA was analyzed on a thick denaturing polyacrylamide gel (see Materials and methods).

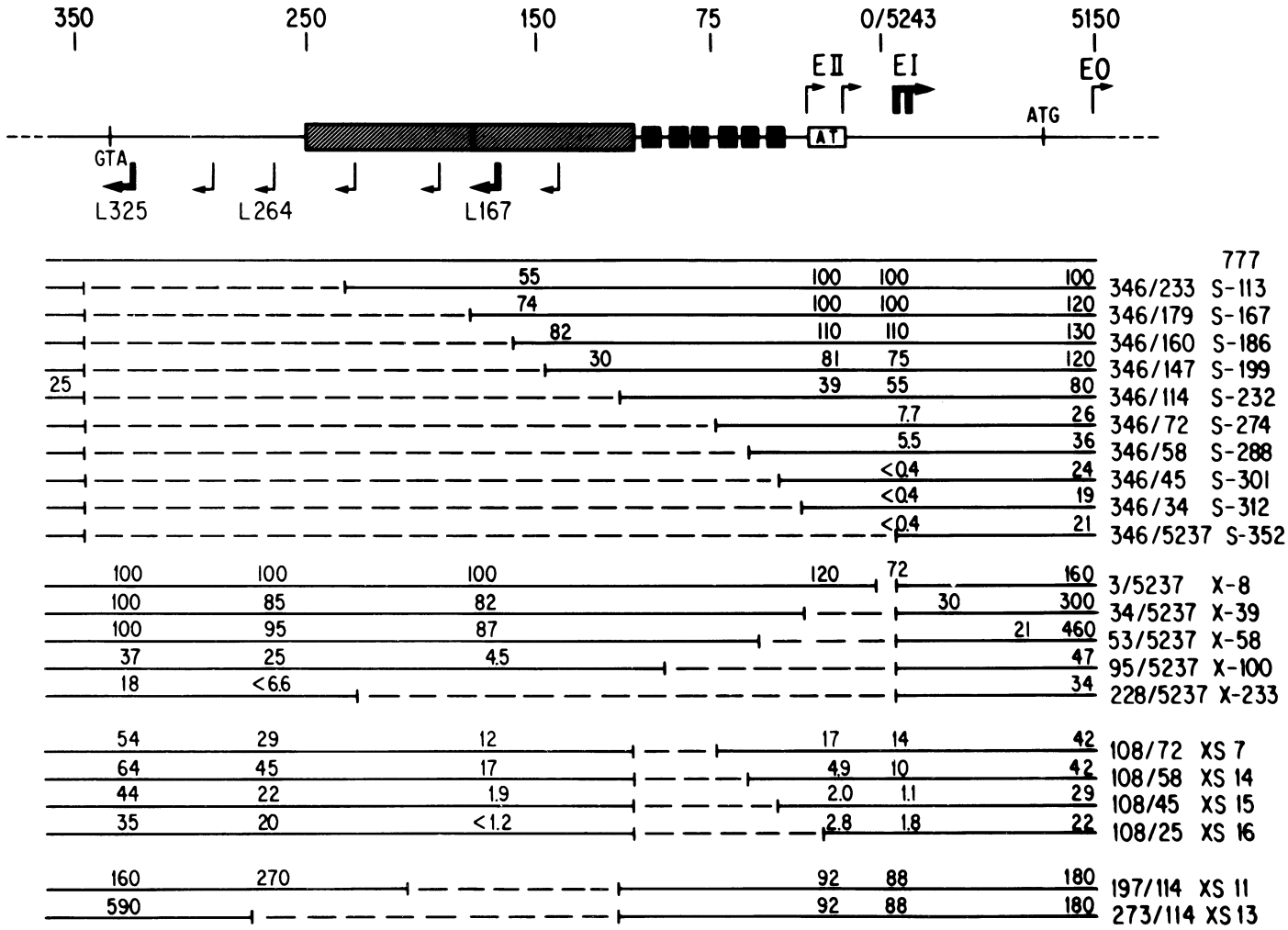


Fig. 5. Scheme of the SV40 early and late promoters and the deletion mutants of interest. The top of the figure indicates a map of the region of the SV40 genome including the late and early promoters. The numbers of the nucleotide positions are given at the top of the figure (Buchman *et al.*, 1981). The hatched boxes represent the two 72-bp direct repeats, and the dark boxes the six PyPyCCGCCC direct repeats. The box labeled AT designates a stretch of 17 A-T base pairs, which contains the early TATA sequence. The ATG at nucleotides 5163 – 5161 encodes the first amino acid for the small (t) and large (T) tumor antigens. The ATG at nucleotides 335 – 337 encodes the first amino acid of the agno gene protein. The various *in vitro* initiation sites of the wild-type early and late promoters are indicated by arrows pointing to the right and left, respectively, with the thicker arrows representing the initiation sites used more prominently. The nucleotide positions of the initiation sites are given in the text. The deletion mutants (Fromm and Berg, 1982) used in the paper are indicated at the bottom, with the deleted sequences represented by dashed lines. The end points of each deletion are given to the right of each line, followed by the name of the mutant. An 8-bp insertion containing the *Sal*I recognition site is present at the deleted regions in the mutants S-113 to S-312, and an 8-bp insertion containing the *Xho*I recognition site in the mutants X-8 to X-233. At the point of the deletion, the XS mutants and S-352 contain 8-bp insertions obtained by combining the *Sal*I and *Xho*I recognition sites of other mutants. Note that the linker sequences in S-301 and XS15 actually reconstruct a CCGCCC sequence, but with the two adjacent 5′ nucleotides being primes, not pyrimidines. 777 is a wild-type strain of SV40, which differs in this region from the wild-type strain SVS (used to construct these mutants) only by a base change at position 5209 (Papamatheakis *et al.*, 1981; and U.Hansen and M.Paskind, unpublished observations). The column of numbers below each initiation site indicates the relative amount of transcription from that site for the various mutant DNA templates, transcribed at high SV40 DNA concentrations (see Table I).

sequences are nearly identical to the SV40 GC repeat and its complement, and since the SV40 repeats act bidirectionally, both would be expected to stimulate transcription in the presence of the SV40-specific factor. In addition, Dierks *et al.* (1983) have recently demonstrated the *in vivo* importance of a similar sequence in the −100 region of the β-globin promoter. Point mutations in the consensus β-globin sequence CCNCACCCTG reduced transcription 3- to 10-fold *in vivo*. This sequence, as the authors noted, shows homology to both the herpes thymidine kinase and SV40 sequences. Dependence on this β-globin sequence for transcription *in vitro* has not been observed, but this may be due to the overriding efficiency of the β-globin TATA sequence. Along this vein, it may be the unique features both of the SV40 TATA sequence(s) and of the GC repeats which allow the demonstration of the strong dependence on upstream sequences for *in vitro* transcription from the SV40 promoters.

Materials and methods

DNA preparations

Plasmid and strain 777 SV40 viral DNAs were purified as described previously (Hansen *et al.*, 1981). Single-stranded M13 DNA was extracted from purified phage.

Transcription

Transcriptions using HeLa whole cell extracts were performed in 25 μl containing 10 μl of extract (Manley *et al.*, 1980) together with the following com-

ponents: 10 mM HEPES (pH 7.5), 40 mM KCl, 5 mM $MgCl_2$, 0.05 mM EDTA, 0.8 mM dithiothreitol (DTT), 7% glycerol, 4 mM creatine phosphate, 350 μM ATP, 100 μM GTP, 100 μM CTP, 5 μM UTP and [α-^{32}P]UTP (ICN), as indicated. The high concentration of ATP optimized initiation at EII (U. Hansen, unpublished observations). The reactions were incubated for 60–75 min at 30°C and extracted twice with phenol/chloroform/isoamyl alcohol and once with chloroform. The nucleic acids were precipitated twice with ethanol in the presence of high concentrations of ammonium acetate.

Transcriptions using the fractionated system (Samuels *et al.*, 1982) contained a mixture of purified RNA polymerase II and three transcriptional factors. The additional components in the reaction mixtures were: 12 mM HEPES (pH 7.9), 4 mM Tris (pH 7.9), 60 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 12% glycerol, and 0.6 mM DTT. Following a 1 h preincubation of factors with DNA (at the indicated concentrations) in 20 μl at 30°C, the transcriptions were initiated by the addition of nucleotides to concentrations of 30 μM ATP, CTP, and UTP and 20 μCi [α-^{32}P]GTP (25 μl volume). Subsequent to a 10 min incubation at 30°C, ATP, GTP, CTP and UTP were added to final additional concentrations of 1 mM (in 30 μl). An additional 10 min incubation at 30°C allowed for elongation of the initiated RNA chains to completion. The details of this preincubation/pulse/chase protocol are described by A. Fire, M. Samuels and P. Sharp (in preparation).

Hybridization selection procedures

RNA samples from transcriptions were resuspended in 15 μl hybridization buffer with 20–25 μg/ml M13 single-stranded viral DNA. The hybridization buffer was 0.75 M NaCl, 50 mM HEPES (pH 7.0) and 1 mM EDTA. The samples were heated for 10 min at 68°C, followed by an incubation at 50°C for at least 1 h. After dilution of the hybrids with 200 μl filtering buffer [10 mM HEPES (pH 7.5), 0.2 M NaCl, 1 mM EDTA (pH 7.2)] the samples were slowly passed through 25 mm nitrocellulose filters (BA85, Schleicher and Schuell) and washed with 1–2 ml more buffer. The nucleic acids were eluted off the filters by heating in 0.5 ml 2 mM EDTA, pH 7.4 at 100°C for 2 min, and chilling quickly to 0°C. Following precipitation with ethanol, the RNA was subjected to gel electrophoresis.

On some occasions, the RNA/M13 DNA hybrids were digested with T1 nuclease prior to filtration. The hybridization reactions were diluted as usual with 200 μl filtering buffer, but then incubated with 2 units T1 nuclease (Sankyo) for 30 min at 30°C. T1 nuclease digests single-stranded RNA 3′ to guanine residues, thus trimming down the hybridized RNA to the guanine residue nearest the end of the hybrid. DNA is not digested. The T1 nuclease was subsequently removed by the addition of 40 μg proteinase K and a 1 h incubation at 30°C. Filtration, elution and gel electrophoresis were carried out as above.

Gel electrophoresis

The RNA samples were analyzed on either thin (0.4 mm) or thick (1.5 mm) 8.3 M urea, 5% polyacrylamide gels. The gels were buffered with Tris-borate-EDTA (Maxam and Gilbert, 1980).

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