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DNA sequences preceding the rabbit β-globin gene are required for formation in mouse L cells of β-globin RNA with the correct 5′ terminus

(eukaryotic cell transformation/deletion mutants/nuclease S1 mapping/reversed genetics/thymidine kinase gene of herpes simplex virus type 1)

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ABSTRACT **Mouse thymidine kinase-negative (TK^-) L cells were transformed with concatenates of cloned herpes simplex virus 1 TK DNA and different rabbit β-globin DNAs in which the globin genes were preceded by native flanking sequences of 14, 66, 76, 425, and 1500 nucleotides. In all cases, selection for TK^+ cell lines led to a high yield of lines producing 5–1500 mature rabbit β-globin-specific RNA strands per cell. The 5′ termini of the transcripts mapped to (*i*) the "cap" nucleotide, (*ii*) positions 42 to 48 nucleotides downstream from the cap site, or (*iii*) positions in the vector DNA preceding the gene. In the case of the gene with only 14 base pairs of 5′ flanking sequence, a high level of rabbit β-globin RNA was produced, but none of the transcripts had the correct 5′ end; most of them originated in the vector moiety. With 66 base pairs of 5′ flanking sequence, 5% of the 5′ termini were correct, and with 76 or more base pairs, 30–85% were correct. The region between 14 and 66 base pairs preceding the cap site contains the Hogness box and appears to be essential for correct initiation of transcription. The region between 66 and 76 base pairs before the cap site contains a variant of the canonical sequence G-G-$\frac{C}{T}$-C-A-A-T-C-T found preceding many other genes at a similar location, and this region may modulate the efficiency of transcription. The sequence of 425 nucleotides preceding the rabbit β-globin gene is reported.**

It has been reported earlier that when thymidine kinase-negative (TK^-) mouse L cells were transformed with cloned rabbit chromosomal β-globin DNA (1, 2) linked to a herpes simplex virus type 1 (HSV1) DNA fragment containing the TK gene (3), 90% of the TK^+ transformants also contained the β-globin gene, frequently in multiple copies (4–6). In the experiments of Mantei *et al.* (6), three-quarters of the cell lines containing β-globin DNA produced rabbit β-globin-specific RNA, of which at least a large fraction was indistinguishable from authentic globin mRNA as regards mobility, poly(A) tail, absence of introns, and correct 5′ terminus; the presence of cap was not ascertained (6).

To determine sequences required for the correct initiation of the β-globin transcripts, we have transformed mouse L cells with hybrid plasmids in which the 5′ flanking sequences of the β-globin gene were deleted to various extents, and we have characterized and quantitated the β-globin RNAs accumulating in the transformed cells.

MATERIALS AND METHODS

Materials. Restriction enzymes *Bsp* I and *Eco*RI were gifts from A. Kiss and W. Boll, respectively; all others were purchased from New England BioLabs. Polynucleotide kinase was purchased from P-L Biochemicals, phage T4 DNA ligase from New England BioLabs, and calf intestine alkaline phosphatase from Boehringer, and terminal deoxyribonucleotidyl transferase and S1 nuclease were gifts from W. Boll and A. Schamböck, respectively.

Construction of Plasmids. *Z-pBR322/RchrβG-Δ425B and Z-pBR322/RchrβG-Δ425C*: Z-pCR1/RchrβG-1 (1) was partially cleaved with *Bgl* II, and the 2070-base-pair (bp) fragment containing the β-globin gene, as well as 425 bp preceding and 350 bp following it, was ligated to *Bam*HI-cleaved pBR322. The orientation of pΔ425B is shown in Fig. 1 *Upper*; pΔ425C has the opposite orientation. *Z-pBR322/RchrβG-Δ14*: pΔ425B was cleaved with *Pvu* II to remove the fragment extending from 9 to about 2115 bp before the cap site of the β-globin gene. The DNA was circularized with T4 DNA ligase and recloned. *Z-pBR322/RchrβG-Δ76*: The *Bsp* I/*Pvu* II (−75 to −9) fragment was isolated from pΔ425B and ligated into the *Pvu* II site of pΔ14. The correct orientation (i.e., the one that reconstituted the correct sequence downstream from −75) was identified by *Pvu* II restriction endonuclease analysis. *Z-pBR322/RchrβG-Δ66*: Z-pCR1/RchrβG-1 was digested with *Hha* I and the fragment containing the entire globin sequence was elongated with dC and joined to dG-tailed pBR322 that had been cleaved with *Bam*HI and *Eco*RI. After cloning, a plasmid was isolated and characterized by restriction endonuclease analysis; the rabbit chromosomal sequence had fortuitously acquired a deletion extending up to position 66 preceding the cap site.

All constructions described above were characterized by restriction endonuclease analysis with *Bsp* I. The lengths of the rabbit DNA sequences preceding the cap site in plasmids pΔ425B, pΔ76, pΔ66, and pΔ14 were determined by sequence analysis (7) and are indicated in Fig. 1 *Lower*.

Transformation of Cells. $LMTK^-$ cells (obtained from P. Goldfarb) were propagated and transformed as described (6), except that plasmids containing the rabbit β-globin gene were linearized with *Sal* I or *Hin*dIII and concatenated with appropriately cleaved Z-pBR322/HSV-TKM2 DNA (3) at a molar ratio of 5:1.

Mapping and Quantitation of Rabbit β-Globin-Specific RNAs in Transformed Cells. Cells ($5–10 \times 10^7$) were lysed with 7.5 ml of 6 M urea/3 M LiCl/200 μg of heparin per ml/10 mM NaOAc (pH 5)/0.1% sodium dodecyl sulfate and RNA was prepared as described (8). Rabbit β-globin-specific RNA was mapped as described by Weaver and Weissmann (9), using DNA

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Abbreviations: TK, thymidine kinase; HSV1, herpes simplex virus type 1; bp, base pair(s).

probes specific for the 3′- and 5′-proximal regions of rabbit β-globin mRNA. Preparation of the 1299-bp *Bgl* II/*Pst* I DNA probe used to assay 3′-proximal rabbit β-globin RNA sequences has been described (6). The DNA probe for mapping 5′-terminal β-globin transcripts was prepared by digesting plasmid pΔ425C with *Mbo* II, dephosphorylating with calf intestine alkaline phosphatase, and isolating the 489-bp fragment spanning the 5′ terminus of the rabbit β-globin gene (position +128 to position −361) by electrophoresis on an 8% polyacrylamide gel containing 90 mM Tris–borate (pH 8.3)/1 mM EDTA. The 5′ termini were labeled with polynucleotide kinase and [γ-^{32}P]ATP (≈9000 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels), and the strands were separated by denaturation in 90% (vol/vol) formamide and electrophoresis in a 5.5% polyacrylamide gel containing 50 mM Tris–borate (pH 8.3) and 1 mM EDTA. The DNA probes (0.03 pmol, 0.4–1.8 μCi/pmol) were hybridized to 50 μg of total cellular RNA as described by Berk and Sharp (10), but at 48°C. After nuclease S1 treatment in siliconized Eppendorf tubes (9) the samples were denatured and analyzed on 6.5% polyacrylamide gels containing 50 mM Tris-borate (pH 8.3), 1 mM EDTA, and 7 M urea. Autoradiograms of gels were made at −70°C by using Fuji medical x-ray film and Ilford fast tungstate intensifying screens.

To obtain a reference for quantitation, the 5′-^{32}P-labeled probe was hybridized to various amounts (0.8–810 pg) of rabbit β-globin mRNA (a gift from T. Stähelin, Basel) and analyzed in parallel with the test samples. Protected DNA fragments were localized by autoradiography and excised, and the Čerenkov radiation was determined. The radioactivity recovered ranged between 2 and 4 cpm/pg of input β-globin mRNA for the 5′ probe and 5–10 cpm/pg for the 3′ probe. In some cases the bands on the autoradiogram were quantitated by densitometry.

Hybridization of Plasmid DNA to the ^{32}P-Labeled 5′ Probe. Plasmids pΔ76, pΔ66, and pΔ14 (0.1 pmol each) were digested with *Hha* I and *Bam*HI; denatured at 80°C in hybridization buffer, and hybridized at 37°C to 0.01 pmol of 5′-^{32}P-labeled minus strand *Mbo* II probe (see above) in a 20-μl reaction mixture containing 50% (vol/vol) formamide, 400 mM NaCl, 40 mM 1,4-piperazinediethanesulfonic acid (pH 6.4), and 0.1 mM EDTA. Hybrids were processed as described above.

RESULTS

The hybrid plasmid used earlier (6) consisted of a 5100-bp fragment of rabbit DNA containing a β-globin gene, inserted in the *Eco*RI site of pCR1 (Fig. 1 *Upper*). In this plasmid the cap site—i.e., the position corresponding to the 5′ end of the globin mRNA—was preceded by about 1500 bp of the rabbit chromosomal DNA. Fig. 1 *Lower* shows the nucleotide sequence of the 425-bp region preceding the cap site; part of this sequence has been presented earlier (2).

To determine whether the entire 1500-bp sequence was essential for transcription, we prepared several plasmids in which segments of DNA preceding the cap site were deleted. All constructions are in pBR322; however, the orientations, modes, and sites of insertion are not all the same, as indicated by the maps of Fig. 1.

The β-globin DNA-containing plasmids were cleaved with *Sal* I or *Hin*dIII and joined to similarly cleaved hybrid plasmid Z-pBR322/HSV-TKM2, which contains the TK gene of HSV1 (3), to give long concatenates (6). TK$^-$ mouse L cells were transformed with this DNA by using the calcium phosphate method (11, 12), and TK$^+$ transformants were selected (13). Three to 12 clones from each experiment were expanded to about 10^8 cells, and RNA and DNA were prepared.

To detect rabbit β-globin-specific DNA sequences, DNA was digested with *Eco*RI and *Hha*I, subjected to agarose gel electrophoresis, transferred to cellulose nitrate membranes, and hybridized to a ^{32}P-labeled rabbit β-globin DNA probe. Two characteristic labeled bands allowed an estimate of the copy number of rabbit β-globin chromosomal sequences. For example, in the case of plasmid pΔ14, all of 12 cell lines gave the expected bands of 639 and 1136 bp. From the relative band intensities we estimate that seven clones had about 1–10 and five clones 10 and more globin DNA copies per cell (data not shown).

Rabbit β-globin-specific RNA was determined by the nuclease S1 procedure (6, 9), using the 1299-bp *Pst* I/*Bgl* II fragment of plasmid pΔ425C, labeled at the 5′ terminus of the *Bgl* II cleavage site, as a probe. In the case of mature mRNA, the 5′-labeled terminus of this probe hybridizes from close to the 3′ end of the coding sequence to the position corresponding to the 3′ edge of the large intron, and S1 cleavage yields a 134-bp labeled fragment (6). If the introns are present in the transcript, a fragment of over 1000 nucleotides is protected. In all analyses, only the 134-nucleotide probe fragment was found (data not shown), showing that no unspliced β-globin RNA accumulated in the cells, as described previously (6). Typically, in transfections with each of the five modified hybrid plasmids 70–100% of the TK$^+$ cell lines showed five or more copies of β-globin-specific RNA per cell. Only 5 of 54 transformed cell lines examined had less than one β-globin RNA copy per cell; four of these were assayed for rabbit β-globin DNA and found to be negative. Among the TK$^+$ cell lines of any one transformation experiment the number of β-globin RNA strands varied quite widely. For example, transformation with plasmid pΔ425B yielded cell lines producing 30–80 globin RNA strands per cell. The variation was greater between two independent, but similar, transformation experiments; for example, in a second experiment with pΔ425B the number of RNA strands per cell ranged between 100 and 500 (data not shown). With the 5′ probe described below, the values were about 20–40 and 100–700, respectively (Table 1).

No correlation between the length of the 5′ flanking region in the β-globin plasmid used for transformation and the amount of globin RNA accumulated was found. Thus, cell lines containing the hybrid plasmid with only 14 bp of 5′ flanking sequence had as much rabbit β-globin-specific RNA (15–1750 copies per cell) as did cell lines containing plasmids with 425 bp of 5′ flanking sequence (8–670 RNA copies per cell; Table 1). However, preliminary experiments showed that in the case of pΔ14 a substantial fraction of globin transcripts originated within the pBR322 moiety. Consequently, it was necessary to determine for each cell line the fraction of globin RNAs possessing the correct 5′ termini.

To determine the 5′ terminus of the β-globin-specific RNA we used the 5′-terminally labeled 489-nucleotide *Mbo* II minus strand probe described in *Materials and Methods*. Fig. 2 *Left* shows that in the case of cell line M2R1, which had been transformed with Z-pCR1/Rchrβ G-1 (6), a plasmid in which the globin gene has a 1500-bp 5′ flanking region, 60% of the 5′ termini of the β-globin-specific RNA mapped at the cap site, while about 40% of the ends mapped to various positions 42 to 48 nucleotides downstream. These downstream termini are shown at higher resolution in Fig. 2 *Right*.

Similar results were found with plasmids in which the β-globin gene is preceded by 76 or 425 bp (Fig. 2 *Left*); in the case of plasmid pΔ425C, the proportion of 5′ termini mapping at the +42 to +48 downstream positions was higher (about 60%) than with the other plasmids. The RNA of some cell lines containing plasmids pΔ425B and pΔ425C protected the probe over its entire length [489 nucleotides (cf. Table 1)]; this protection

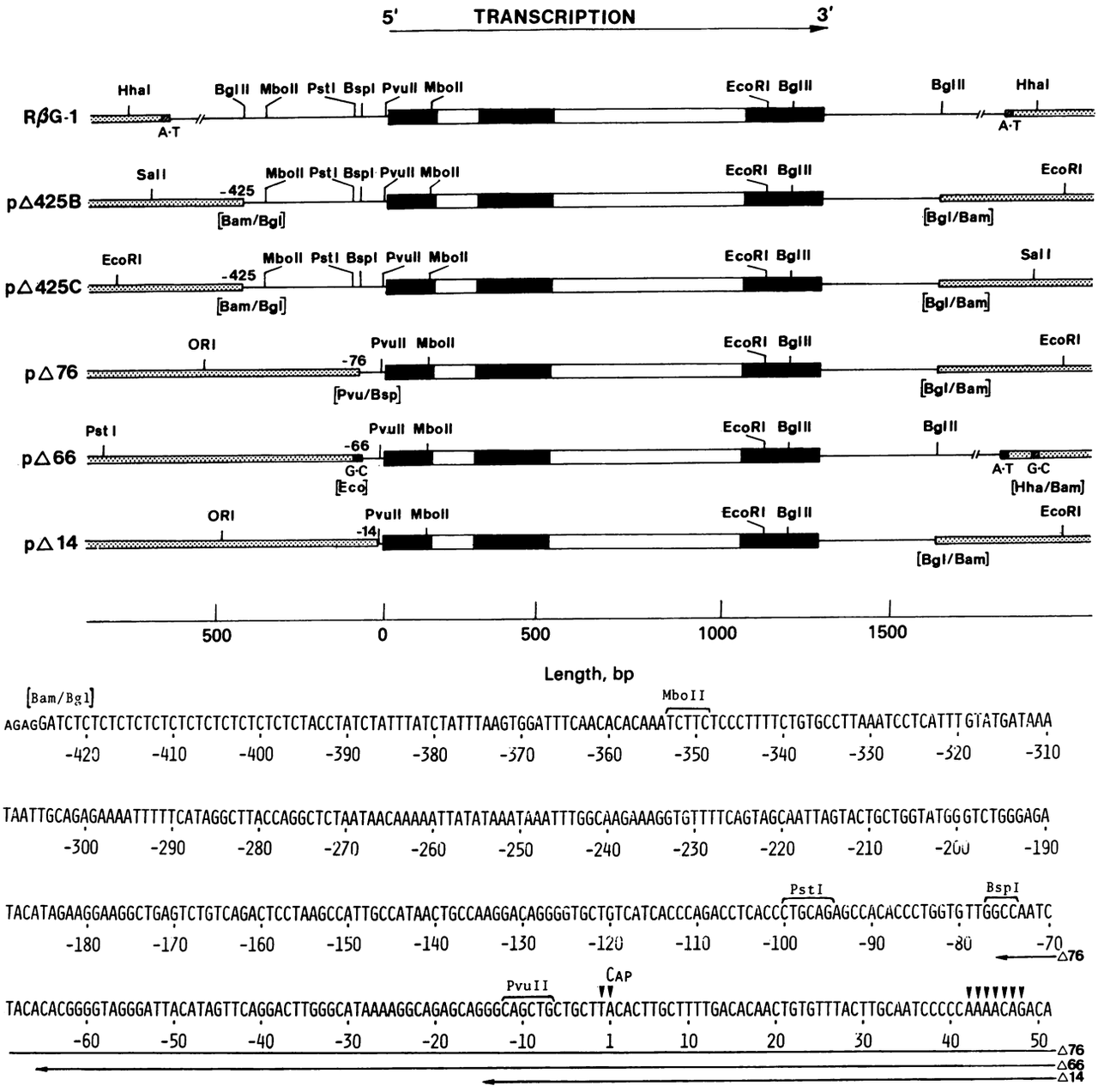

FIG. 1. Restriction site analysis and partial sequence of rabbit β-globin plasmids. (*Upper*) Restriction site map of the globin gene and surrounding regions of plasmids used in this study. Black boxes, globin gene exons; white boxes, intervening sequences; solid lines, nonglobin rabbit DNA; stippled boxes, plasmid sequences; hatched boxes, A·T or G·C tails, ORI, origin of replication. Restriction sites in brackets were fused. Only the *Bsp* I and *Mbo* II sites used for preparation of plasmids or probes are shown. The orientation of the globin can be identified by the restriction sites in the plasmid sequences. In RβG-1 the plasmid sequences are from pCR1 (1). The inserts in pΔ76 and pΔ14 have the same orientation as in pΔ425B; pΔ425C and pΔ66 have the opposite one. (*Lower*) Sequence around the transcription initiation site of the rabbit β-globin gene. The sequence downstream from −224 has been published (2). The upstream segment was determined in pΔ425B from the *Mbo* II site at −360 bp and from the nearest *Hha* I site in pBR322. Large letters, rabbit sequences; small letters, plasmid sequences. Position 1 corresponds to the cap site. Horizontal arrows indicate sequences present in plasmids pΔ76, pΔ66, and pΔ14. The downward-pointing arrowheads indicate the positions to which the "correct" and "incorrect" (downstream) 5′ termini map, as deduced from the data presented in Fig. 2 *Right*, taking into account the nucleotide shift described (9).

is ascribed to the presence of transcripts originating in the pBR322 moiety.

The proportion of transcripts with correct 5′ termini is only about 5% in the cells transformed with plasmid pΔ66, which has a 5′ flanking region 10 nucleotides shorter than that of plasmid pΔ76; most termini (about 70%) map in the +42 to +48 downstream region (Fig. 2 *Left*, Table 1). Although the proportion of correct 5′ termini is low, all cell lines producing β-globin RNA contained some RNA with the correct 5′ terminus. In contrast, cells transformed with plasmid pΔ14 contained no globin RNA with the correct 5′ terminus, although several cell lines containing this plasmid had more than 1000 copies of β-globin-specific RNA per cell.

DISCUSSION

Only about 60% of the rabbit β-globin transcripts in the transformed mouse cells have the correct 5′ termini, even when a

Table 1. Copy number and 5′ termini of rabbit β-globin RNA in mouse L cell lines transformed with rabbit β-globin DNA plasmids carrying different deletions

Plasmid*	Exp.†	β-Globin-RNA⁺ colonies/ TK⁺ colonies	β-globin RNA in rabbit β-globin DNA-transformed cell lines,‡ copies per cell	% 5′ termini mapping at:§ Upstream	Cap site	+42 to +48
Z-pCR1/RchrβG-1 (1500 bp)		Cell line M2R1	235	ND	62	38
pΔ425B (425 bp)	1	3/3	22–44	4 (0–14) [0–7]	49 (27–72) [39–56]	46 (30–63) [41–54]
	2	6/10	97–670	13 (0–30)¶ [0–29]	48 (34–62)¶ [35–61]	39 (28–49)¶ [28–50]
pΔ425C (425 bp)	1	8/8	8–115	8 (0–16) [0–23]	31 (22–40) [24–51]	61 (46–75) [32–81]
pΔ76 (76 bp)	3	10/10	9–240	ND	86 (78–94) [63–95]	14 (6–22) [5–37]
pΔ66 (66 bp)	1	10/11	15–130	21 (16–27) [14–37]	5 (2–8) [1–12]	73 (67–79) [57–81]
pΔ14 (14 bp)	2	12/12	15–1750	71 (66–76) [58–83]	ND	29 (24–34) [17–42]

The 5′ termini were determined as described in *Materials and Methods*. The mean distribution of 5′ termini in the sample, $\bar{x}$, as well as the 95% confidence limits of the mean of the population, μ, were calculated. For statistical analysis raw percentage data were first transformed to data having an underlying normal distribution by using the arcsine transformation (equation 14.3 in ref. 14); the means and confidence limits, calculated as degrees, were then transformed back to percentages.

* Values in parentheses indicate the length in bp of rabbit chromosomal DNA preceding the 5′ end of the gene.

† Ligations and transfections conducted in parallel are indicated by the same experiment number.

‡ One picogram of rabbit β-globin-specific RNA per 50 μg of total cellular RNA is estimated to be equivalent to 1.3 copies per cell (6). Copy numbers are based on the amount of β-globin-specific RNA as assayed with the 5′ probe. Only cell lines containing detectable β-globin RNA are included.

§ The 95% confidence limits for the population mean are in parentheses. The range of percentage values is in brackets. ND, none detected. The limit of detection is about 1 copy of β-globin RNA per cell.

¶ One clone was not included in these calculations because it yielded aberrant results.

long 5′ flanking region is present; the other 5′ termini map to a position 42–48 bp downstream from the cap site. These downstream sites correspond to those reported by Wold *et al.* (5) as the only termini for rabbit β-globin transcripts in transformed mouse cells. It is not known whether these termini are the result of incorrect initiation or nucleolytic cleavage. In addition, transcripts containing the β-globin sequence are initiated from pBR322 regions flanking the chromosomal DNA moiety of the plasmid (Fig. 2 *Left*, Table 1).

Clearly, the evaluation of the effect of 5′ flanking rabbit sequences of various lengths on transcription must take into account primarily the globin mRNA molecules with correct 5′ termini. The data of Table 1 show that the plasmids containing the β-globin gene preceded by 66 or more bp of 5′ flanking rabbit DNA can support correct initiation of transcription, whereas a plasmid (pΔ14) containing only 14 bp of 5′ flanking sequence cannot. This suggests that at least part of the sequence between nucleotides −14 and −66 (which contains the so-called Hogness box) is essential for initiation at the cap site.

To quantitate the effect of the deletions on the efficiency of transcription we have calculated the absolute number of correct transcripts per cell. Because it varied widely among the cell lines generated in any one experiment, this measurement does not yield a useful criterion for comparing correct initiation on the different plasmids. If we assume that the 5′ termini mapping downstream of the cap site reflect an inherent, relatively weak initiation point for transcription, at least in mouse L cells, then these may be used as an internal reference to which correct starts can be related. Two experiments involving the same plasmid, pΔ425B, gave very different absolute numbers of correct starts (average 16 and 115); however, the ratios of correct to downstream starts were quite similar, 1.1 and 1.2, respectively. The plasmid pΔ425C, which differs from pΔ425B only by the orientation of the globin DNA insert, also showed a similar ratio, 0.5 (data not shown). In contrast, the ratio for plasmid pΔ66 was 0.07 (compared to a ratio of less than 0.01 in the case of pΔ14), whereas the ratio for pΔ76 was 6. The difference between pΔ66 and pΔ76 is particularly striking, especially because Benoist *et al.* (15) have recognized a variant of the canonical sequence G-G-$\frac{C}{T}$-C-A-A-T-C-T 69–77 bp upstream from the cap site in the 5′ flanking sequence of the rabbit β-globin gene, and in similar locations in several other genes, such as chicken ovalbumin, conalbumin, and others (15). It may be that this sequence, like the one preceding the Pribnow box in *Escherichia coli* promoters, is required for efficient initiation of transcription (16). In evaluating our results, it must be borne in mind that the different deletion mutants have different plasmid sequences flanking the 5′ end of the rabbit DNA, and that these may also modulate the efficiency of transcription. Clearly, the use of point mutations to define regions of functional importance is much to be preferred (17, 18).

The importance of the Hogness box region for accurate initiation of transcripts was shown by Grosschedl and Birnstiel, who examined the expression of sea urchin H2A genes injected into *Xenopus* oocytes: deletion of the T-A-T-A sequence gave rise to new transcriptional start points of lower efficiency, whereas deletion of the natural mRNA start point gave rise to transcripts initiating 24 nucleotides downstream from the T-A-

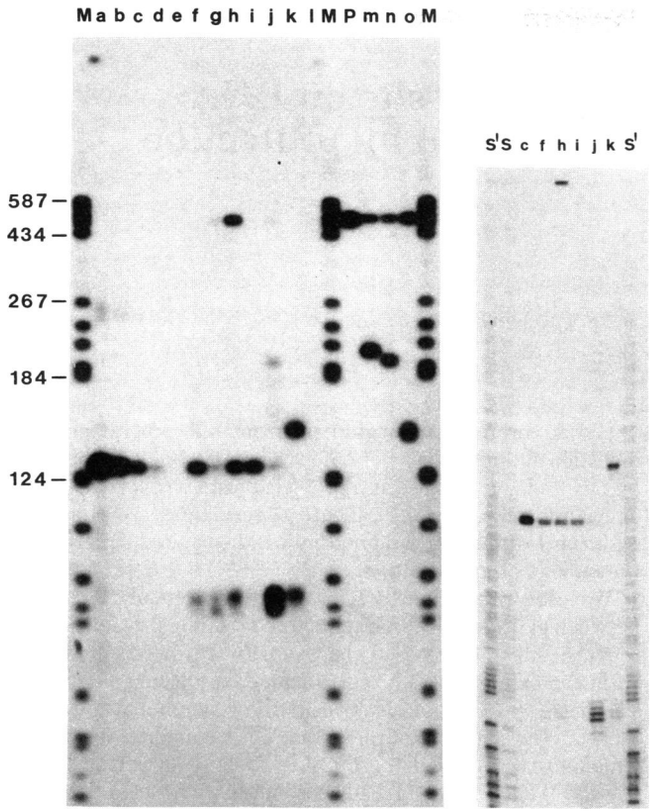

FIG. 2. S1 nuclease mapping of rabbit β-globin-specific RNA in transformed mouse cells. Total cellular RNA (50 μg) was hybridized to 0.024 pmol (0.45 μCi of [^{32}P]phosphate per pmol) of the 5′-^{32}P-labeled "5′ probe" and treated with S1 nuclease, and the products were electrophoresed on 6.5% (*Left*) or 12% (*Right*) polyacrylamide gels. The sources of the RNA (and the amount of RNA from which the loaded sample was derived) are as follows. (*Left*) Lanes a–e: 270, 80, 27, 8, and 0 pg, respectively, of authentic rabbit β-globin mRNA; lane f: Z-pCR1/Rchrβ G-1-transformed cell line M2R1 (25 μg); lane g: cell line Δ425C-297 (50 μg); lane h: cell line Δ425B-122 (10 μg); lane i: cell line Δ76-102 (20 μg); lane j: cell line Δ66-107 (100 μg, from a double reaction); lane k: cell line Δ14-104 (5 μg); lane l: cell line transformed with Z-pBR322/HSV-TKM2 alone (50 μg). (*Right*) Lanes as in *Left*. To provide markers for the identification of transcripts initiated upstream of the rabbit DNA inserts, the 5′ probe was hybridized to pΔ76, pΔ66, or pΔ14 DNA that had been digested with *Eco*RI and *Hha* I (*Left*, lanes n, o, and p, respectively) and digested with S1 nuclease. Size markers: M, pBR322 digested with *Bsp* I and 5′-^{32}P-labeled; P, untreated probe; S, 5′ probe subjected to A+G Maxam–Gilbert degradation (7); S′, mixture of 5′ probe subjected to A+G degradation and 5′ probe subjected to C+T degradation.

T-A box (19). More recent experiments with this system have shown that sequences upstream of the Hogness box dramati cally affect the efficiency of transcription (20).

The requirements for correct initiation of gene transcripts have been studied *in vitro* by several groups. Using a set of cloned DNAs with deletions of various extents, Chambon and his colleagues have found that sequences from −10 to −44 and from −12 to −32 (21, 22), both of which contain the Hogness box, play an essential role in specific transcription of the conalbumin and adenovirus late transcription units, respectively. Moreover, a point substitution within the Hogness box reduced *in vitro* transcription of the conalbumin gene to at least 1/10th (23). So far, no evidence has been published showing the requirement for sequences in the region of about −70 nucleotides from the cap site for optimal transcription *in vitro*. Apart from the possibility that precise quantitation may be required to reveal such an effect, it may be that crude *in vitro* systems using naked DNA are not adequate for these analyses.

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