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DNA Sequences Required for Regulated Expression of <latex>β</latex>-Globin Genes in Murine Erythroleukaemia Cells

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DNA sequences required for regulated expression of β-globin genes in murine erythroleukaemia cells

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We have introduced into murine erythroleukaemia (MEL) cells a series of human globin gene cosmids and two sets of hybrid genes constructed from the human β-globin gene and the human γ-globin or murine H-2K^{bm1} genes. S1-nuclease analysis of the mRNA products from these genes before and after MEL cell differentiation showed that the human β-globin gene, but not the human ε- or γ-globin or H-2K^{bm1} genes, is induced specifically. Hybrid genes containing human β-globin DNA sequences from either 5′ or 3′ side of the translation initiation site were both inducible. Measurement of the relative rate of transcription showed this induction to be the result of transcriptional activation. We therefore suggest that DNA sequences which regulate β-globin gene expression during MEL differentiation are located both 5′ and 3′ to the translation initiation site.

Introduction

The human β-like globin genes are differentially expressed during the course of development. The five active genes which are located in a cluster on the short arm of chromosome 11 are expressed at different times and in different erythroid tissues. The embryonic ε-globin gene is expresed in the yolk sac, the foetal ^{G}γ- and ^{A}γ-globin genes in the foetal liver, and the adult δ- and β-globin gene in adult bone marrow (for review, see Maniatis *et al.* 1981). To study this tissue and stage specific expression, we have used DNA mediated gene transfer into cultured murine erythroleukaemia (MEL) cells. Chemically induced differentiation of these cells results in an increase in transcription by a factor of several hundred of the adult mouse globin genes (Friend *et al.* 1971; Ross *et al.* 1972; Reuben *et al.* 1976; Marks & Rifkind 1978). These cells thus serve as a model for gene activation during erythropoiesis and can be used to study the expression of foreign β-globin genes, as first shown by Willing *et al.* (1979) and Pyati *et al.* (1980). They used human chromosome 11-MEL cell hybrids to show that expression of the human β-globin, but not the human ε- and γ-globin genes, is regulated in such MEL cell hybrids. MEL cells were subsequently used to study the expression of foreign globin genes introduced by DNA mediated gene transfer (Spandidos & Paul 1982; Wright *et al.* 1983; Chao *et al.* 1983). We have used a series of cosmid DNA containing the human ε-, γ- and β-globin genes to show that the expression of the β-globin, but not the ε- and γ-globin genes, is regulated during MEL cell differentiation. These were followed by a series of recombinant genes with parts of the human β-globin gene to localize the sequence involved in the regulated expression.

Regulated expression of the human β-globin gene

A series of recombinant cosmids containing different regions of the human β-globin locus (figure 1) was introduced into thymidine kinase negative (tk^-) MEL cells (Spandidos & Paul 1982). Before the calcium phosphate transformation (Wigler *et al.* 1979), the DNA was linearized within the vector sequences with PvuI. Between one and twenty stable tk^+ transformants per 10^6 cells per microgram DNA were obtained after selection in HAT medium.

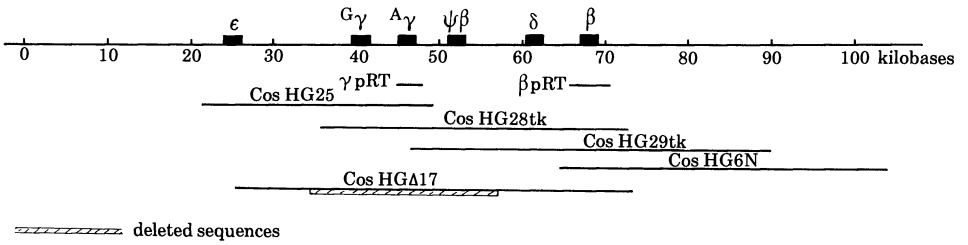

Figure 1. Structure of human globin gene cosmids. The human globin cluster was cloned in cosmid vectors containing the HSV *tk* gene (Grosveld *et al.* 1981; Grosveld *et al.* 1982). Cosmid DNA was prepared by the method of Birnboim & Doly 1979). PvuI linearized cosmid DNA was introduced into tk^- MEL cells (Spandidos & Paul 1982) by calcium phosphate transformation (Wigler *et al.* 1979) and selection in hypoxanthine-aminopterin-thymidine (HAT) medium. Reprinted by permission from *Nature*, vol. 305, no. 5932, pp. 333–336.

Each transformant was shown to contain 1–15 copies of non-rearranged exogenous DNA by Southern blot analyses (not shown). Ten different transformants from each cosmid transformation were induced to differentiate by culturing for three days in the presence of 3 mM hexamethylene bisacetimide (HMBA). RNA was prepared from each culture (Auffrey & Rougeon 1980) before and after differentiation. The levels of human and mouse globin RNA transcripts were quantitated by S1 nuclease protection (Berk & Sharp 1977; Weaver & Weissmann 1979) by using probes from the 5′ and 3′ ends of the genes.

Of the 54 clones tested, 52 showed more than a 100-fold increase in the levels of mouse β-major globin mRNA as measured by S1 nuclease protection analysis with a probe corresponding to the 3′ end of the gene (figure 2*a*). This corresponds to more than 20000 copies of mRNA per induced cell. Six of the transformants that showed induction had high levels (over 1000 copies per cell) of mouse β-major globin mRNA before induction.

Of 30 transformants that contained a foreign human β-globin gene, 23 showed an increase in the level of human β-globin mRNA after induction (average 40- to 50-fold), as shown in figure 2*b* with a probe corresponding to the 3′ end of the human β-globin gene. The induced levels of human β-globin mRNA corresponded to 150–2000 copies per cell. Of the seven transformants that did not induce, one also showed no induction for the endogenous mouse globin gene, and four showed high levels of mRNA before induction by HMBA. The levels of human β-globin mRNA either before or after induction did not correspond to the number of β-globin genes present in the transformants. Seventeen of nineteen transformants and all of nine transformants containing the complete human γ-globin gene (figure 2*c*), or the human ε-globin gene (not shown) showed the same or a smaller amount of γ- or ε-globin mRNA respectively. Two γ-globin transformants showed a low inducibility of γ-globin mRNA.

These data with cosmid recombinants strongly suggest that the large flanking sequences are not required for the observed regulated expression of the β-globin gene. Regulation is obtained

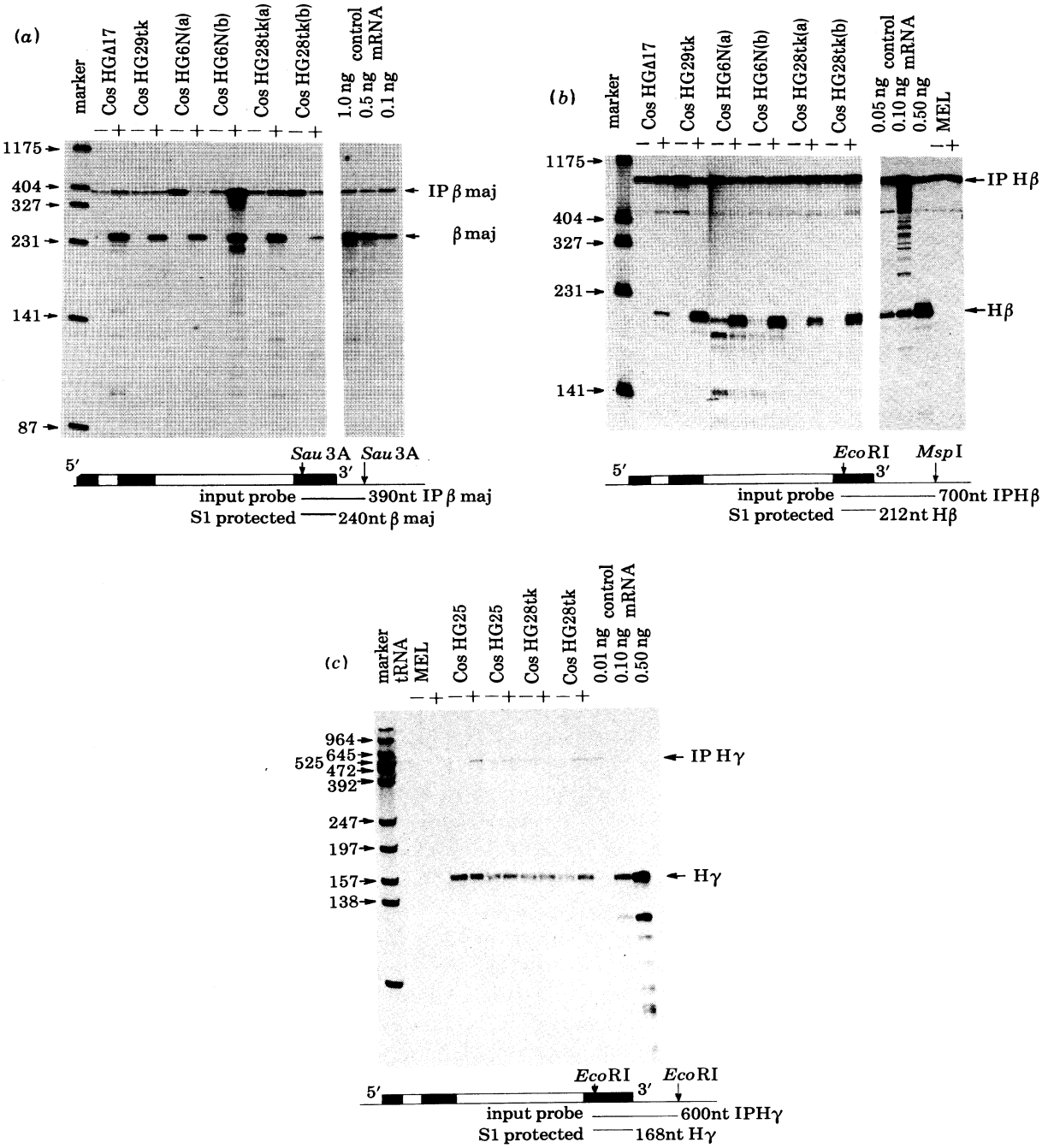

FIGURE 2. (*a*) S1 nuclease analysis of mouse β-major globin mRNA in MEL cell transformants. Control RNA was obtained from mouse reticulocytes. (*b*) S1 nuclease analysis of human β-globin cosmid containing MEL transformants (figure 1). Control RNA was obtained from a patient with sickle cell anaemia. (*c*) S1 nuclease analysis of human γ-globin cosmid containing MEL transformants (figure 1). Control RNA was obtained from a patient with $\beta^0/\delta\beta^0$ thalassaemia (Moschonas *et al.* 1981). MEL transformants were grown for three days in the absence (−) or presence (+) of HMBA, and RNA was prepared (Auffrey & Rougeon 1980). The S1 nuclease analysis was as described by Wright *et al.* (1983). Reprinted by permission from *Nature*, vol. 305, no. 5932, pp. 333–336. Copyright © 1983 Macmillan Journals Ltd.

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for both Cos HG28tk and Cos HG6N, which only contain approximately 2 kilobases of 3′ flanking DNA and 5 kilobases of 5′ flanking DNA sequences. To confirm these results a second series of MEL cells was selected after transformation with βpRT, γpRT and pTME10. These plasmids contain the human β-globin gene, the human γ-globin gene and the murine major histocompatibility gene H-2K^{bm1} respectively (Weiss *et al.* 1983) and short flanking sequences at the 5′- and 3′- ends of the genes (figure 3).

Eight out of nine transformants with the human β-globin gene (β*pRT*) showed the same increased levels of β-globin mRNA (figure 3*a*, *b*) as the transformants described above, when analysed with 5′- or 3′- and S1 nuclease protected probes. In contrast, only one of twelve γ-globin gene transformants and none of twelve H-2K^{bm1} gene transformants showed any increase in the respective mRNA levels (figure 3*c*, *d*). These results indicate therefore that only the β-globin gene expression is regulated in MEL-cells and that the regulatory DNA sequences appear to be located within a BglII fragment of 4.7 kilobases spanning the gene.

Localization of the sequences required for regulated expression

To determine whether the human β-globin DNA sequences that confer inducibility upon the gene are located in the 5′ promoter region, two hybrid genes were constructed (figure 4). The 5′ end of the human β-globin gene (up to the translation initiation site) was adjoined to the complementary 3′ region of either the human γ-globin or mouse H-2K^{bm1} genes, both of which are non inducible (see above).

The hybrid genes were introduced into tk^{-} MEL cells and individual transformants isolated. Five out of ten 5′β–3′γ-globin transformants showed a 5- to 50-fold induction of the mRNA level as measured with a probe that detects the 3′ end of the γ-globin gene (figure 5*a*). In the second set, two out of three 5′β–3′H-2K hybrid transformants showed a similar induction, as measured with a probe specific for the H-2K mRNA (figure 5*b*).

A second set of hybrid genes was constructed to determine whether any inducible sequences were located to the 3′ site of the initiation codon of the β-globin gene. In these cases, the complementary constructs to those described above were made, i.e. the γ-globin or the H-2K 5′ end of the genes were adjoined to the body of the β-globin gene. The construction of these 5′H-2K–3′β and 5′γ–3′β hybrid genes is shown in figure 6. Both sets of genes were introduced into tk^{-} MEL cells by co-transfer with *pRT*. S1 nuclease protection, with a 3′β-globin specific probe showed that four out of eight 5′γ–3′β and four out of five 5′H-2K–3′β hybrid gene mRNA levels increased at least five to more than 100-fold after induction (figure 7*a*, *b*). It has been shown that the accumulation of mouse globin mRNA in differentiating MEL cells is not only the result of an increase in the rate of transcription, but also of an increase in the relative stability of globin mRNA. It is therefore possible that the increase in the mRNA levels of the 5′γ–3′β and the 5′H-2K–3′β hybrid genes may be due to an increase in the stability of the mRNA rather than to an increase in the role of transcription. To distinguish between these possibilities, we measured the relative rate of transcription of these genes before and after differentiation using *in vitro* nuclear run-off transcription (Groudine *et al.* 1981; Hofer *et al.* 1982). Nuclei were isolated from MEL cells containing the hybrid genes, both before and after differentiation. RNA was extracted from these nuclei after a nuclear transcription reaction in the presence of ^{32}P-UTP and hybridized to globin probes spotted on to nitrocellulose filters. Three DNA probes were used, an insulin probe (Bell *et al.* 1981) as a negative control and two globin probes from the large IVS of the mouse and human β-globin genes respectively. These probes therefore measure

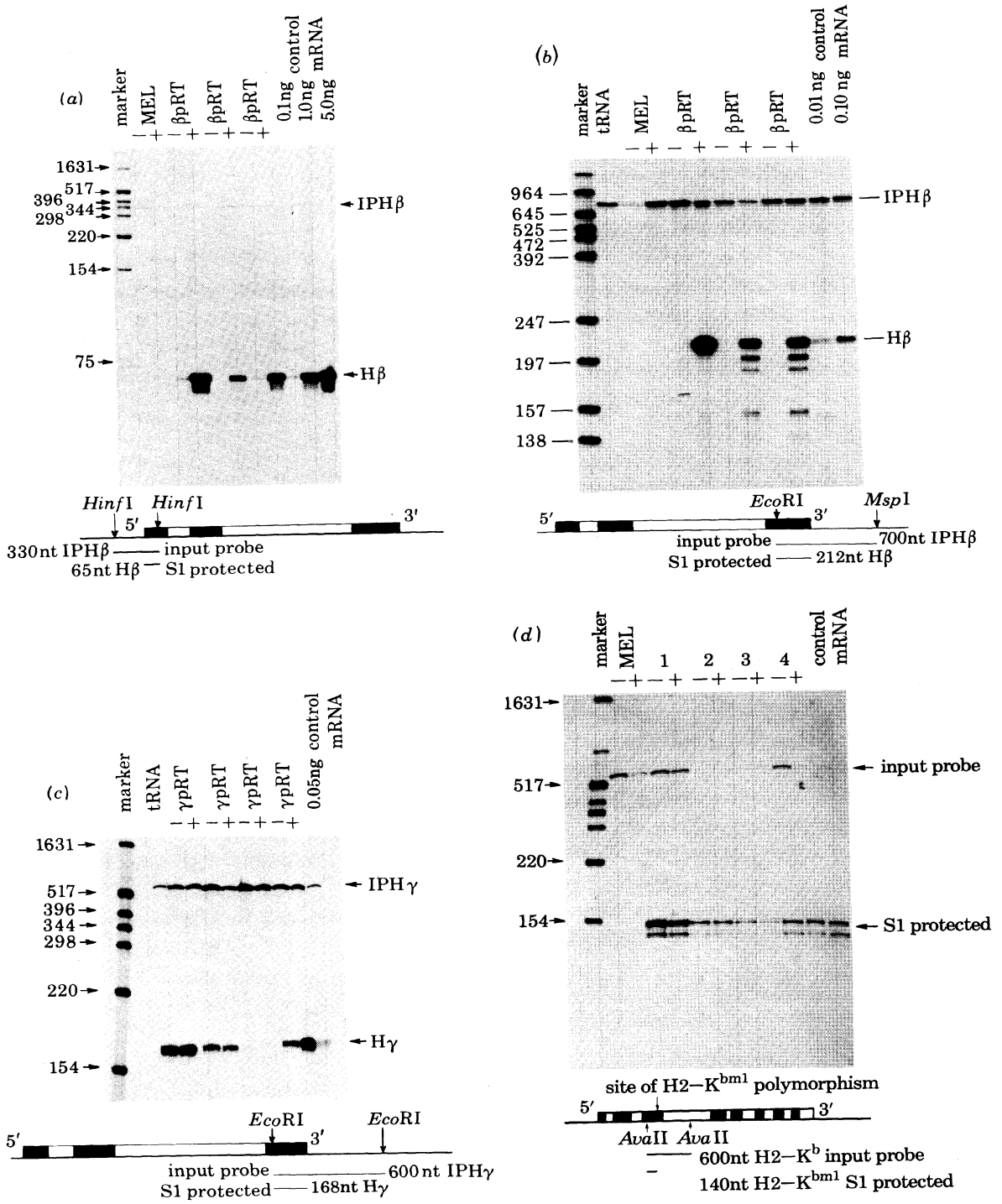

FIGURE 3. (*a*), (*b*) S1 nuclease analysis of MEL transformants containing β *pRT* with a 5′ S1 nuclease probe and a 3′ S1 nuclease probe, respectively. Controls and methods as in figure 2. (*c*), (*d*) S1 nuclease analysis of MEL cell transformants containing γ *pRT* or the H-2K^{bm1} gene respectively. Control RNAs were as in figure 2 and mouse spleen mRNA respectively. The methods were as described in figure 2. Reprinted by permission from *Nature*, vol. 305, no. 5932, pp. 333–336. Copyright © 1983 Macmillan Journals Ltd.

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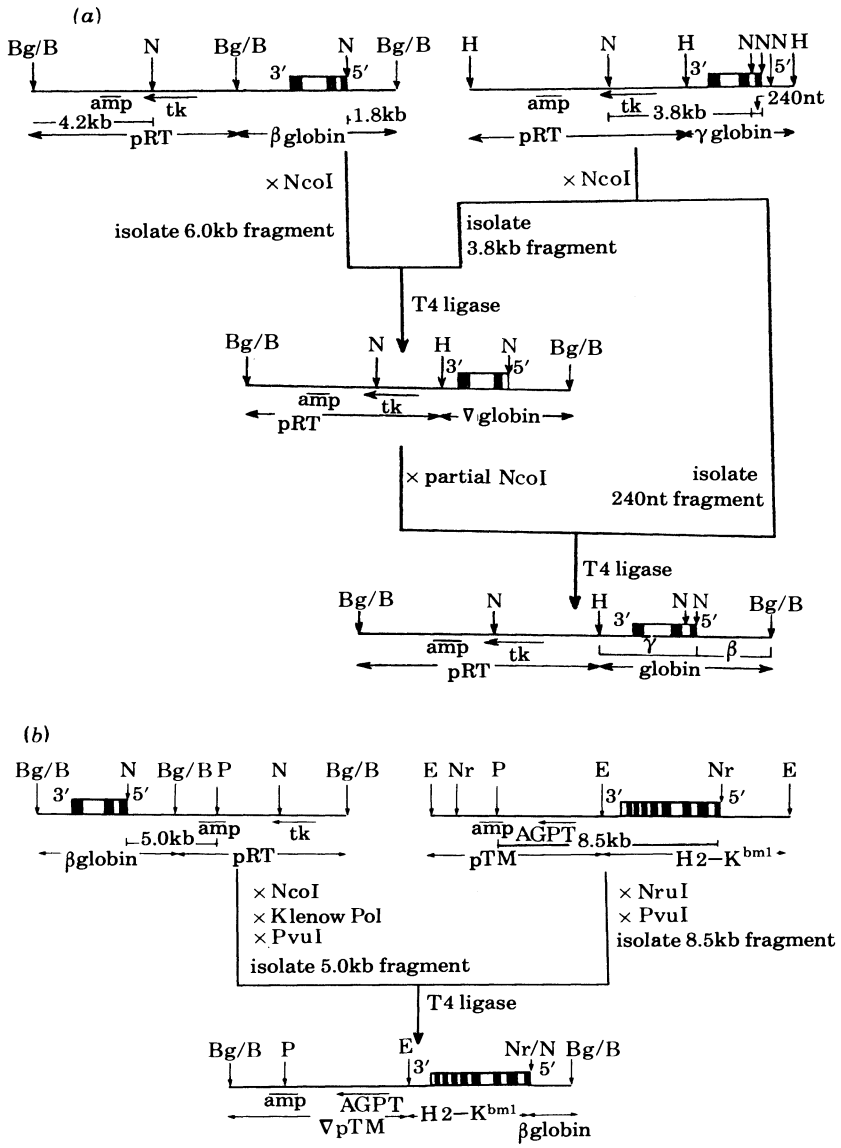

FIGURE 4. Construction of (*a*) 5′β–3′γ and (*b*) 5′β–3′H-2K^{bm1} hybrid genes. The hybrid genes were constructed from the globin and H-2K^{bm1} genes as described in the diagrams. N, P, E, Nr refer to *Nco*I, *Pvu*I, *Eco*RI and *Nru*I sites respectively. kb, Kilobases.

the amount of precursor mRNA in an area of the greatest sequence divergence between the mouse and human globin genes. The hybridization contained RNA from an equal number of nuclei and a vast excess of filter bound DNA probe, so that differences in the absolute rate of gene transcription could be measured.

Differentiation of control untransformed MEL cells showed an increase in hybridization to the mouse probe, but not the human probe after differentiation (figure 8). This indicated an

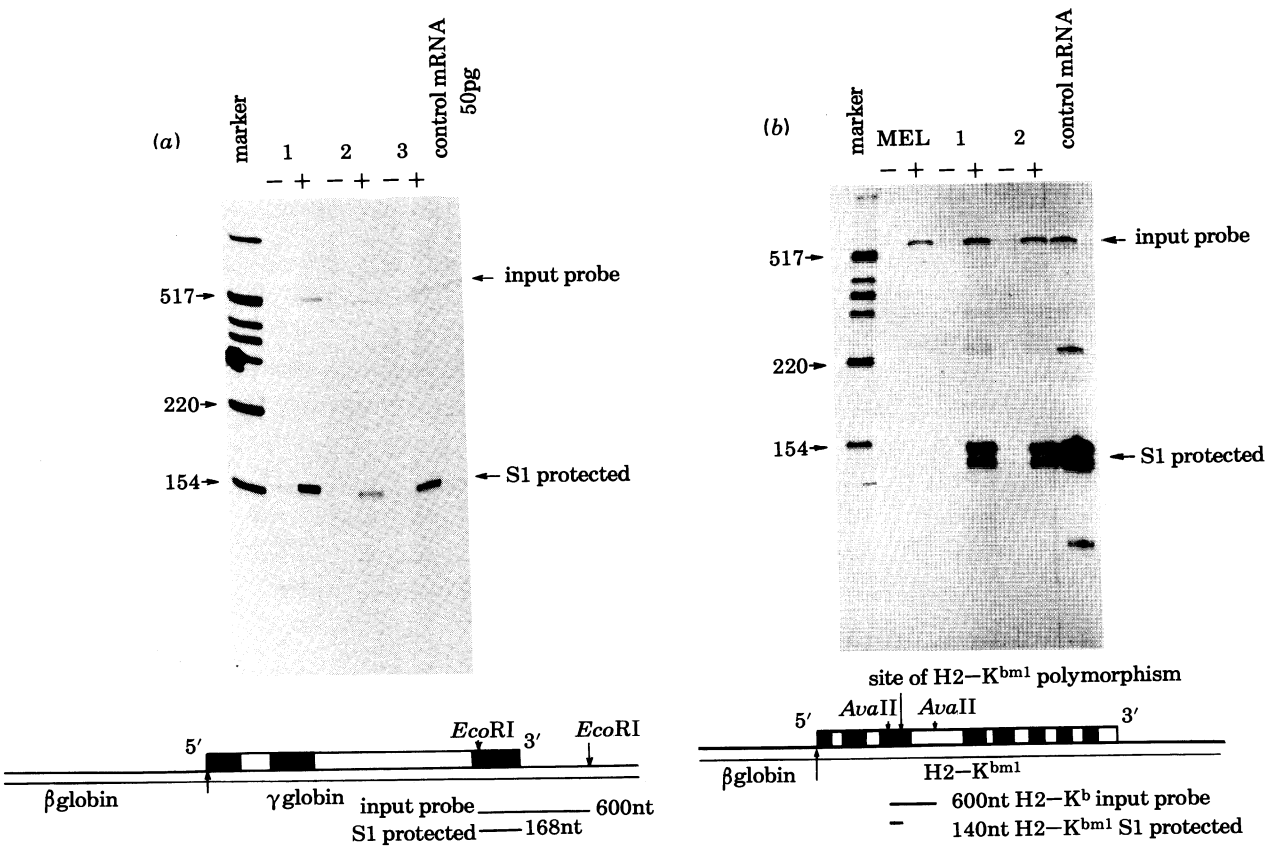

FIGURE 5. (*a*) S1 nuclease analysis of MEL cell transformants containing 5′β–3′γ hybrid globin genes. (*b*) S1 nuclease analysis of MEL cell transformants containing 5′β–3′H-2K^{bm1} globin genes. Control RNAs and methods were as described in figures 2 and 3.

increase in the rate of transcription of the mouse β-globin mRNA, which did not cross hybridize with the human probe. In contrast, MEL cells containing the 5′γ–3′β or 5′H-2K–3′β hybrid genes showed an increased hybridization to both the mouse and human probes. Thus, the increase in the level of 3′β hybrid gene transcripts resulted (at least in part) from an increase in the rate of transcription following MEL differentiation.

DISCUSSION

We have shown independent regulation in the expression of individual human ε-, γ- and β-globin genes when introduced into MEL cells as a gene cluster. In accordance with the results obtained with human chromosome 11-MEL cell hybrids (Willing *et al.* 1979; Pyati *et al.* 1980), the expression of human adult, but not foetal or embryonic genes, is regulated during MEL cell differentiation. In our case, all the genes are expressed constitutively, probably because the genes are linked to a selective marker. The marker gene (*tk*) in turn will be integrated in an active part of the MEL cell genome and be expressed constitutively to grow under selective conditions. Different integration sites and chromatin structures of the introduced genes in

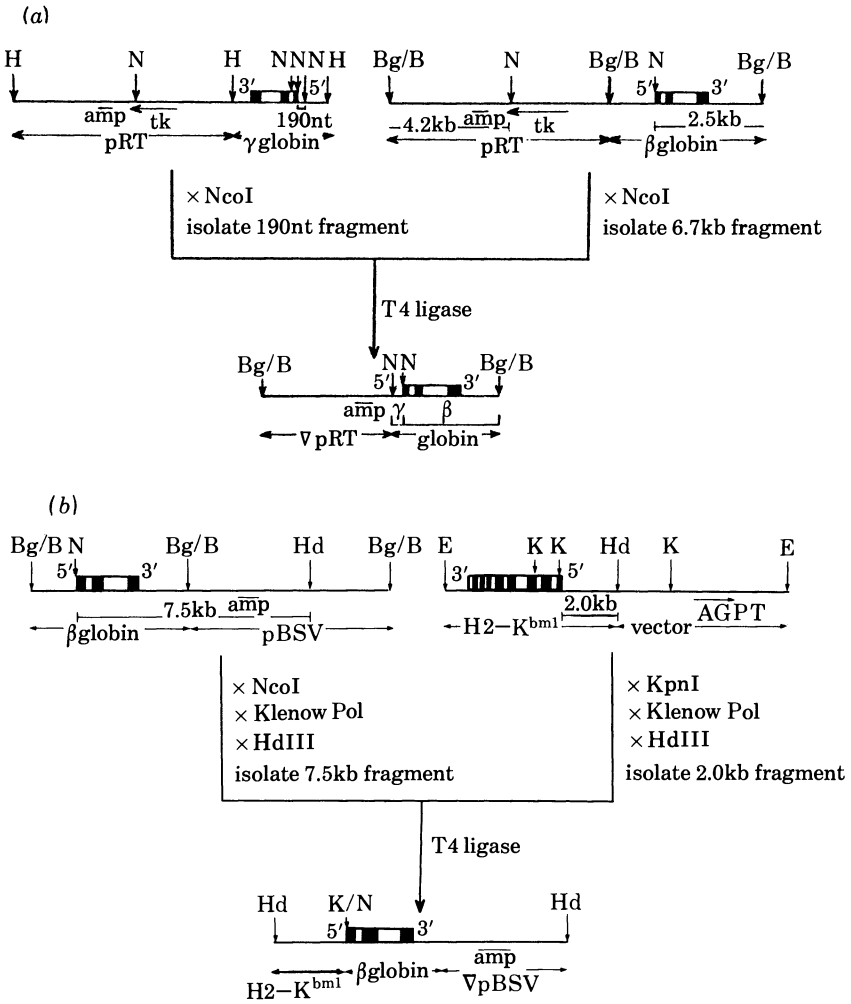

FIGURE 6. Construction of (*a*) 5′γ–3′β and (*b*) 5′H-2K^{bm1}–3′β genes. The genes were constructed from globin and H-2K^{bm1} plasmids as described in the diagram. N, R, E, K refer to *Nco*I, *Pvu*I, *Eco*RI and *Kpn*I sites respectively.

separate transformants, rather than any heterologous effects would also explain the variable levels of expression and induction of the introduced genes (Wright *et al.* 1983; Chao *et al.* 1983). To define the DNA region of the β-globin gene involved in induction, we constructed two types of hybrid genes: one set containing the upstream sequences from the initiation codon of the β-globin gene linked to the downstream complementary part of two non inducible genes (5′β–3′γ and 5′β–3′H-2K). The mRNA levels of these hybrid genes was increased after MEL cell differentiation, which suggests that the 5′- flanking upstream region of the β-globin gene contains sequences that confer inducibility of transcription.

A second set of hybrid genes contained the upstream region from the non inducible γ-globin and H-2K genes linked to the part of the β-globin gene downstream from the initiation codon (5′γ–3′β and 5′H-2K–3′β). Surprisingly, we also observed an increased level of transcripts from these hybrid genes after MEL cell differentiation. Nuclear run-off analysis showed this increase to be (at least partly) due to an increase in the rate of transcription. We therefore suggest that DNA sequences required for the activation of the β-globin gene during erythropoiesis are also

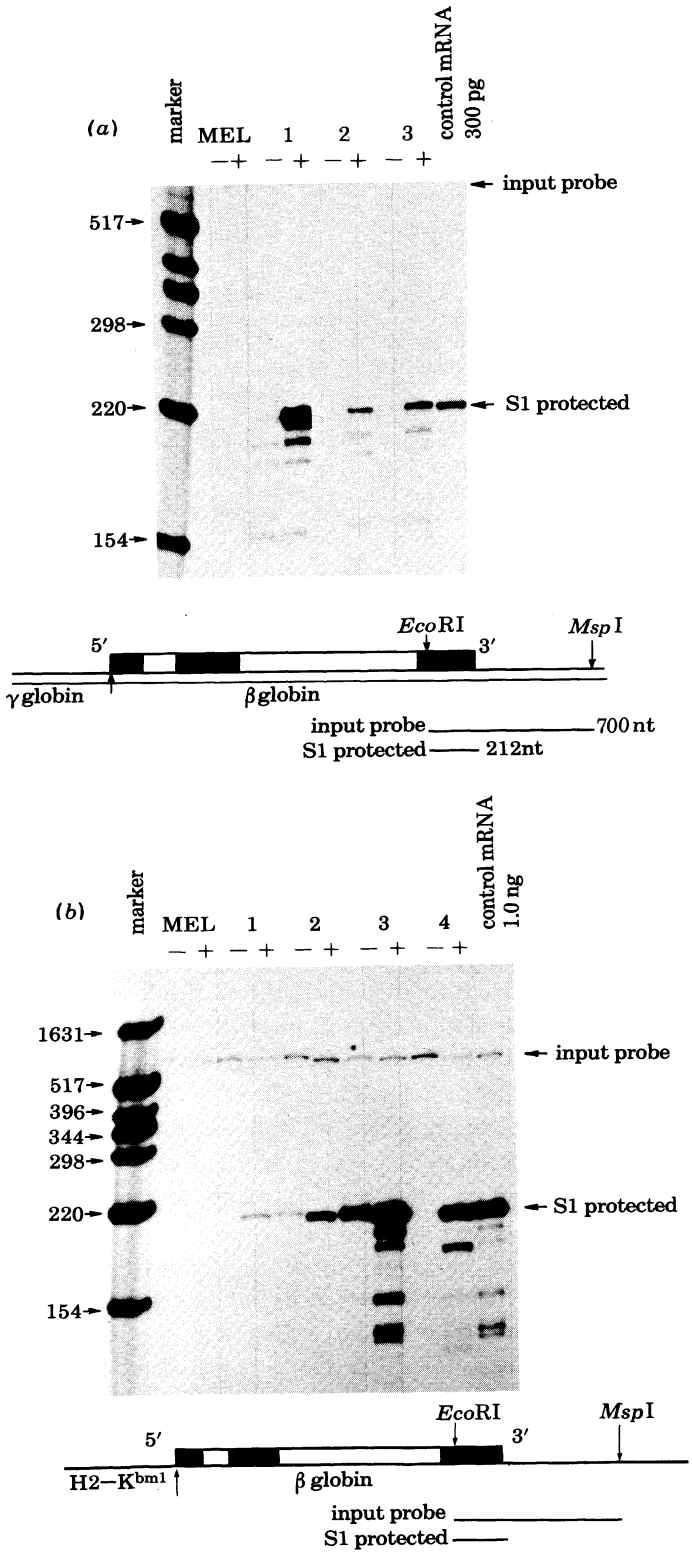

FIGURE 7. (*a*), (*b*) S1 nuclease analysis of the 5′γ–3′β and 5′H-2K^{bm1}–3′β hybrid genes respectively. Control RNA and methods were as described in the previous figures.

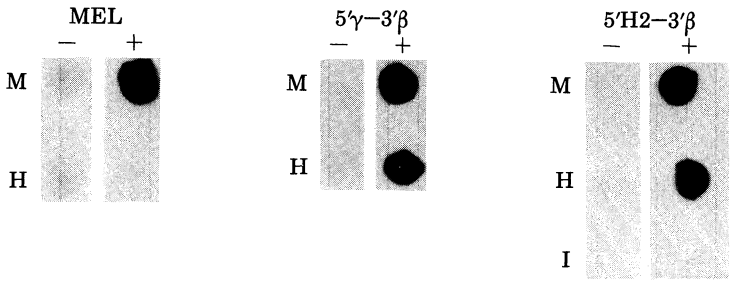

FIGURE 8. Nuclear run-off transcription of MEL cell transformants containing hybrid globin genes. Nuclei were isolated from non-induced (−) and induced (+) MEL cell transformants containing a 5′γ–3′β or a 5′H-2K^{bm1}–3′β hybrid gene. RNA was labelled with ^{32}P-UTP (300 μCi, 3000 Ci/mmol per 5×10^7 nuclei) as described by Groudine *et al.* (1984). Two μg of purified DNA fragments were bound to a 0.45 μm nitrocellulose filter, as described by Kafatos *et al* (1979), and hybridized with labelled nuclear transcript as described by Groudine *et al.* (1984). Each hybridization contained nuclear RNA from 10^7 cells which corresponded to 5×10^7 to 10×10^7 counts per minute for the non-induced cells and 2×10^7 to 5×10^7 for the induced cells. After hybridization, the filters were washed to a stringency of 0.3 × SSC. The human β-globin (H), mouse β-major globin (M) and human insulin (I) fragment probes were an *Eco*RI–*Bam*HI IVS fragment, a *Hin*III–*Pst*I IVS fragment and a 1.8 kilobases *Bam*HI fragment (Bell *et al.* 1982), respectively.

located at the 3′ side of the translation initiation codon. This would also explain the fact that the rabbit β-globin gene is still induced upon differentiation when large regions of the upstream sequences have been deleted (Wright, unpublished observations).

There are several other examples of gene transcription influenced by DNA sequences located downstream of the camp site, e.g. the internal promoter region of the *Xenopus* 5*S* RNA gene and the tissue specific enhancer of the immunoglobulin gene, which is located in an intron. In addition, it has been shown that the expression of foreign genes in eukaryotic cells can be increased by a rival enhancer when it is placed either at the 5′- or 3′- side of the gene.

Specific binding of the glucocorticoid receptor to MTV DNA *in vitro* occurs at sites both within and upstream of the transcribed region. This suggests that DNA sequences both within and upstream of the gene may regulate this glucocorticoid responsive gene. The results described in this paper may similarly indicate that DNA sequences both within and upstream of the globin gene may play a role in their activation during erythroid differentiation. Whether the activation, is accomplished by the binding of a positive stimulatory factor(s), or the removal of a negative repressive factor(s) supplied *in trans* by the MEL cell during differentiation, is not clear from these results. The low levels of expression obtained with the 5′β-globin construct 5′β–3′H-2K (figure 7) suggest that the β-globin promoter might be very weak or repressed in undifferentiated MEL-cells, although these results cannot be quantified reliably. Nevertheless, it is tempting to suggest that both a negative regulation would operate on the 5′ end and a positive regulation on the 3′ end of the β-globin gene.

The evidence to date suggests that the induction *in trans* could only operate *in vivo* after the β-globin chromatin structure has been activated during erythroid differentiation. Changes in globin chromatin structure have been shown to occur with globin gene activation in chicken (Groudine *et al.* 1981) and humans (Groudine *et al.* 1983). Similarly, in MEL cells, new DNaseI hypersensitive sites appear in the mouse β major globin region after induction of MEL cell differentiation (Hofer *et al.* 1982; Sheffery *et al.* 1982). Although not proven, this suggests a causal relationship between globin gene activation and changes in chromatin structure. This relationship has been used to explain the phenotype of one form of γβ-thalassaemia (van der

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Ploeg *et al.* 1979; Kouissis *et al.* 1983). In these patients a large deletion has removed the normal upstream sequences and positioned an unknown locus next to the β-globin gene. When this β-globin gene is cloned and introduced into MEL cells, it is expressed and induced normally (Wright, unpublished observations). Consequently, because this abnormal β-globin locus can respond normally to MEL cell differentiation, and because the heterozygous patient also contains a normal and expressed β-globin allele, it suggests that factors which act *in trans* could only act *in vivo* after the activation of the globin chromatin structure.

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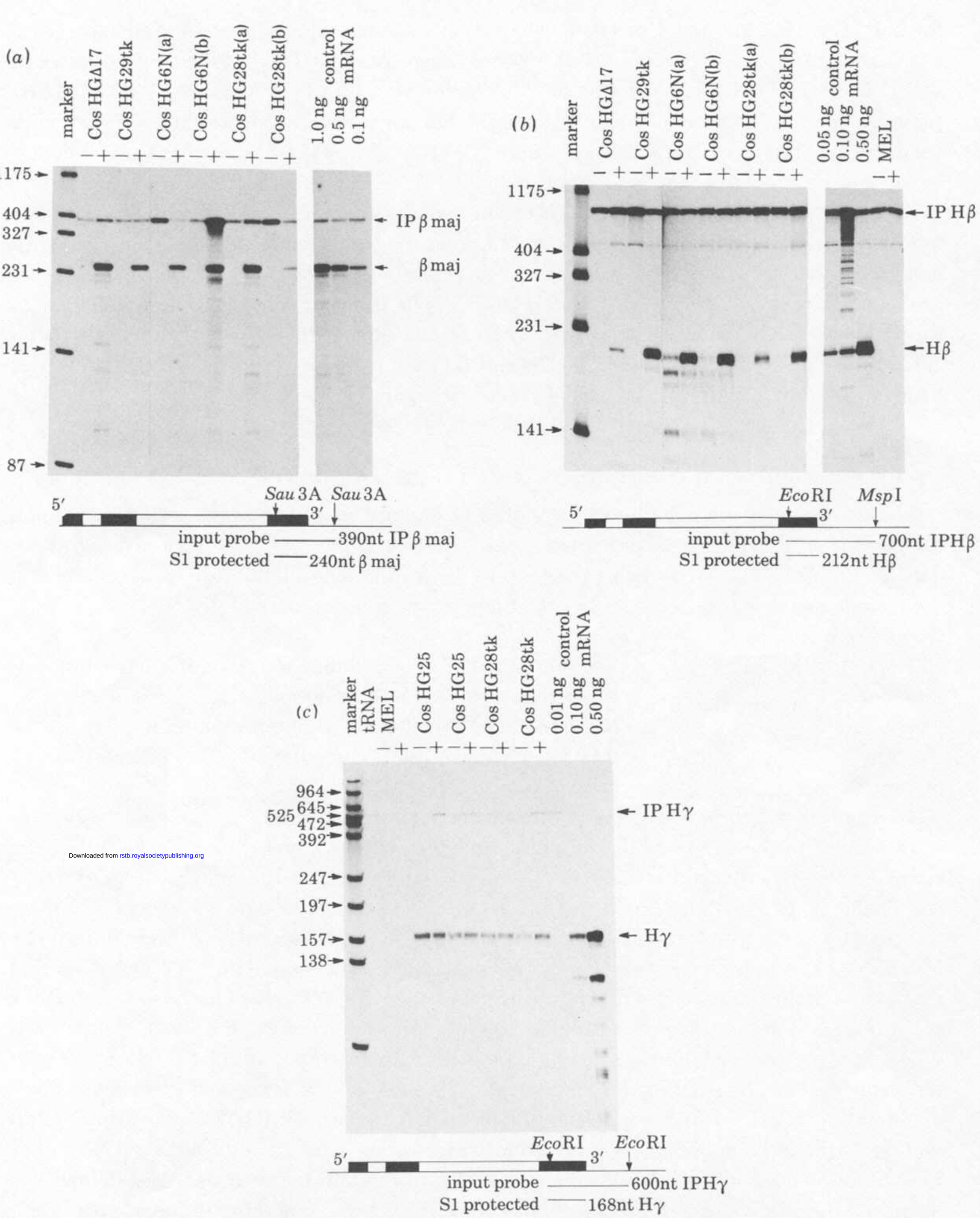
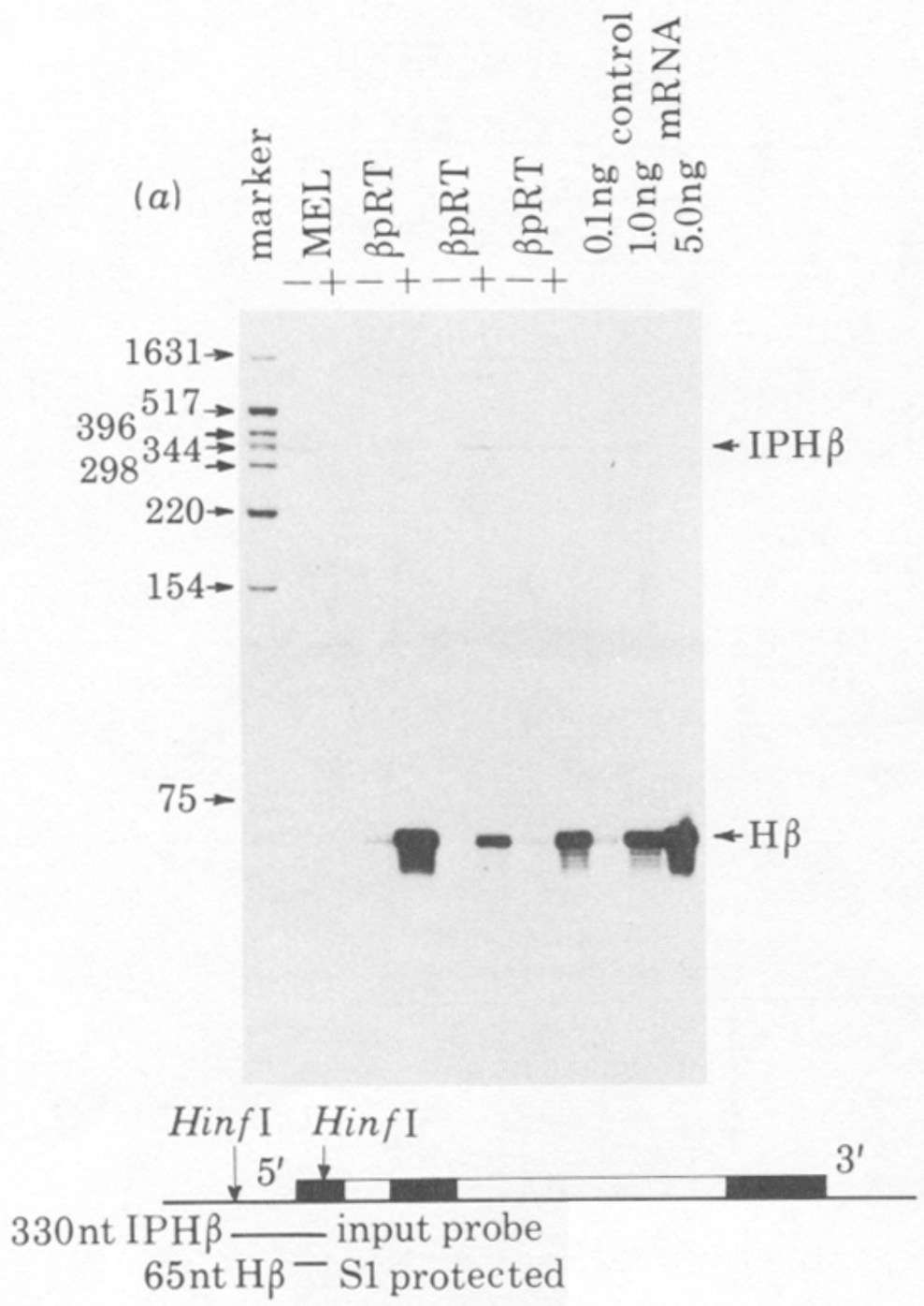
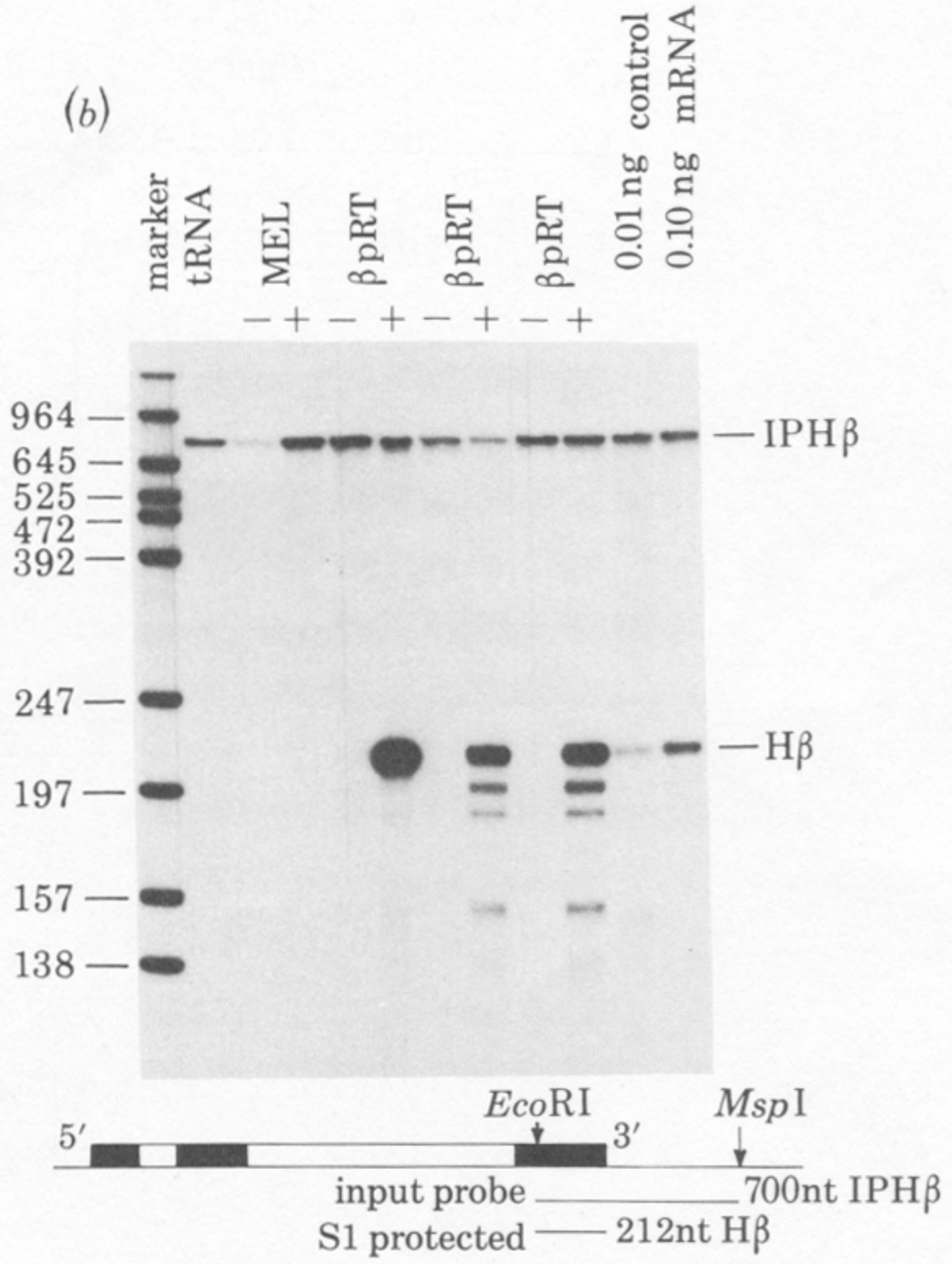
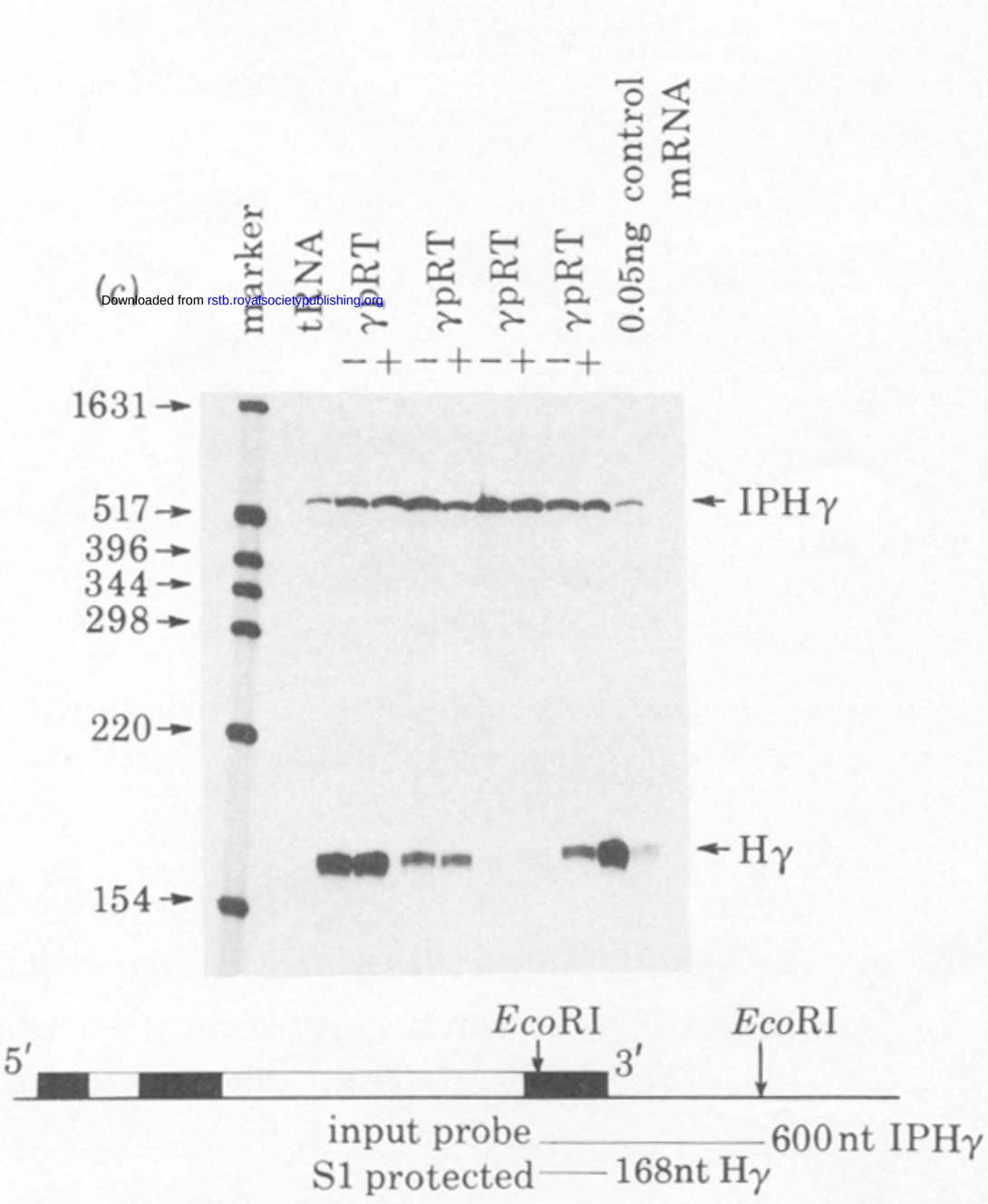


FIGURE 2. (*a*) S1 nuclease analysis of mouse β-major globin mRNA in MEL cell transformants. Control RNA was obtained from mouse reticulocytes. (*b*) S1 nuclease analysis of human β-globin cosmid containing MEL transformants (figure 1). Control RNA was obtained from a patient with sickle cell anaemia. (*c*) S1 nuclease analysis of human γ-globin cosmid containing MEL transformants (figure 1). Control RNA was obtained from a patient with $\beta^0/\delta\beta^0$ thalassaemia (Moschonas *et al.* 1981). MEL transformants were grown for three days in the absence (−) or presence (+) of HMBA, and RNA was prepared (Auffrey & Rougeon 1980). The S1 nuclease analysis was as described by Wright *et al.* (1983). Reprinted by permission from *Nature*, vol. 305, no. 5932, pp. 333–336. Copyright © 1983 Macmillan Journals Ltd.







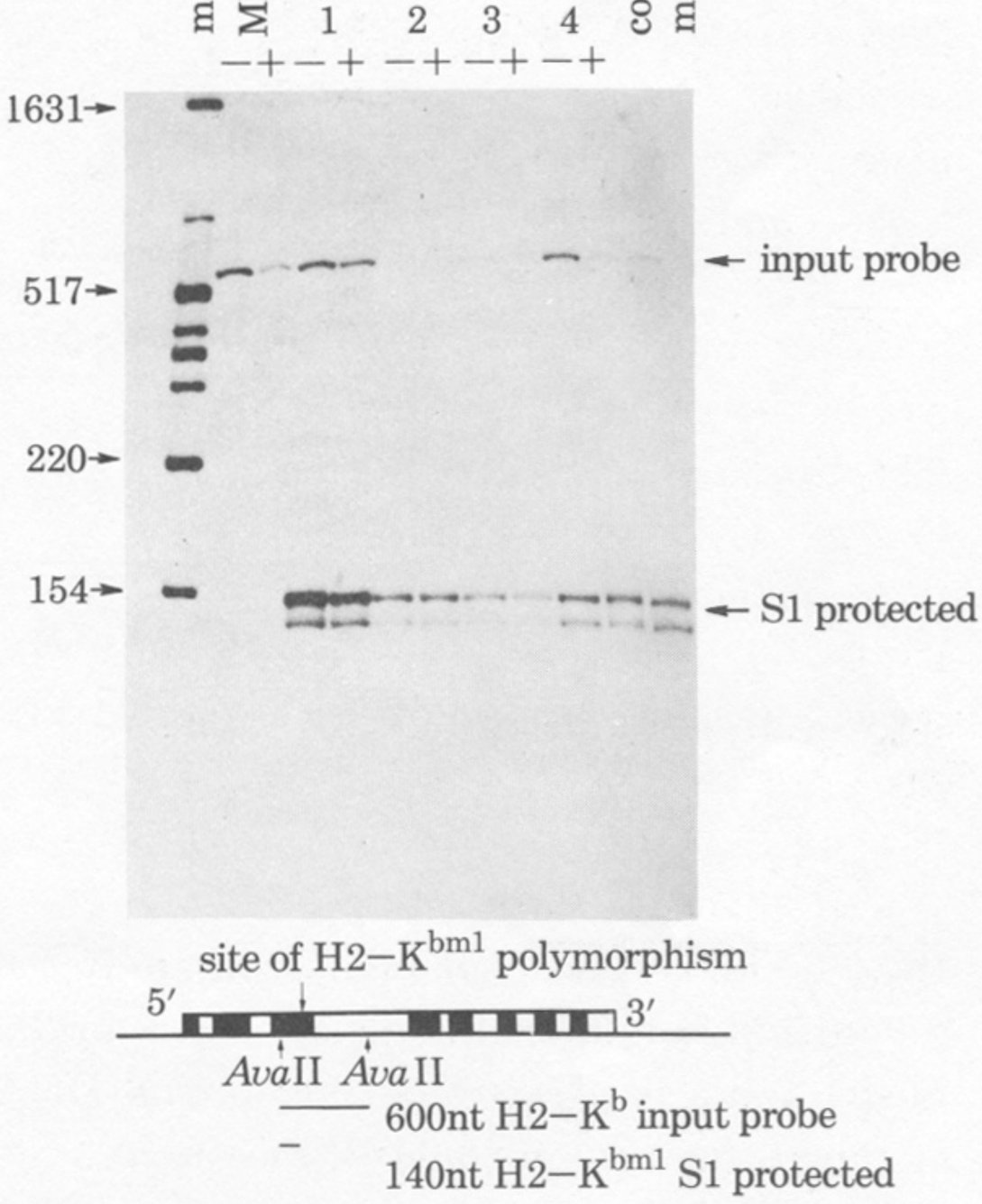


FIGURE 3. (*a*), (*b*) S1 nuclease analysis of MEL transformants containing β *pRT* with a 5′ S1 nuclease probe and a 3′ S1 nuclease probe, respectively. Controls and methods as in figure 2. (*c*), (*d*) S1 nuclease analysis of MEL cell transformants containing γ *pRT* or the H-2K^{bm1} gene respectively. Control RNAs were as in figure 2 and mouse spleen mRNA respectively. The methods were as described in figure 2. Reprinted by permission from *Nature*, vol. 305, no. 5932, pp. 333–336. Copyright © 1983 Macmillan Journals Ltd.

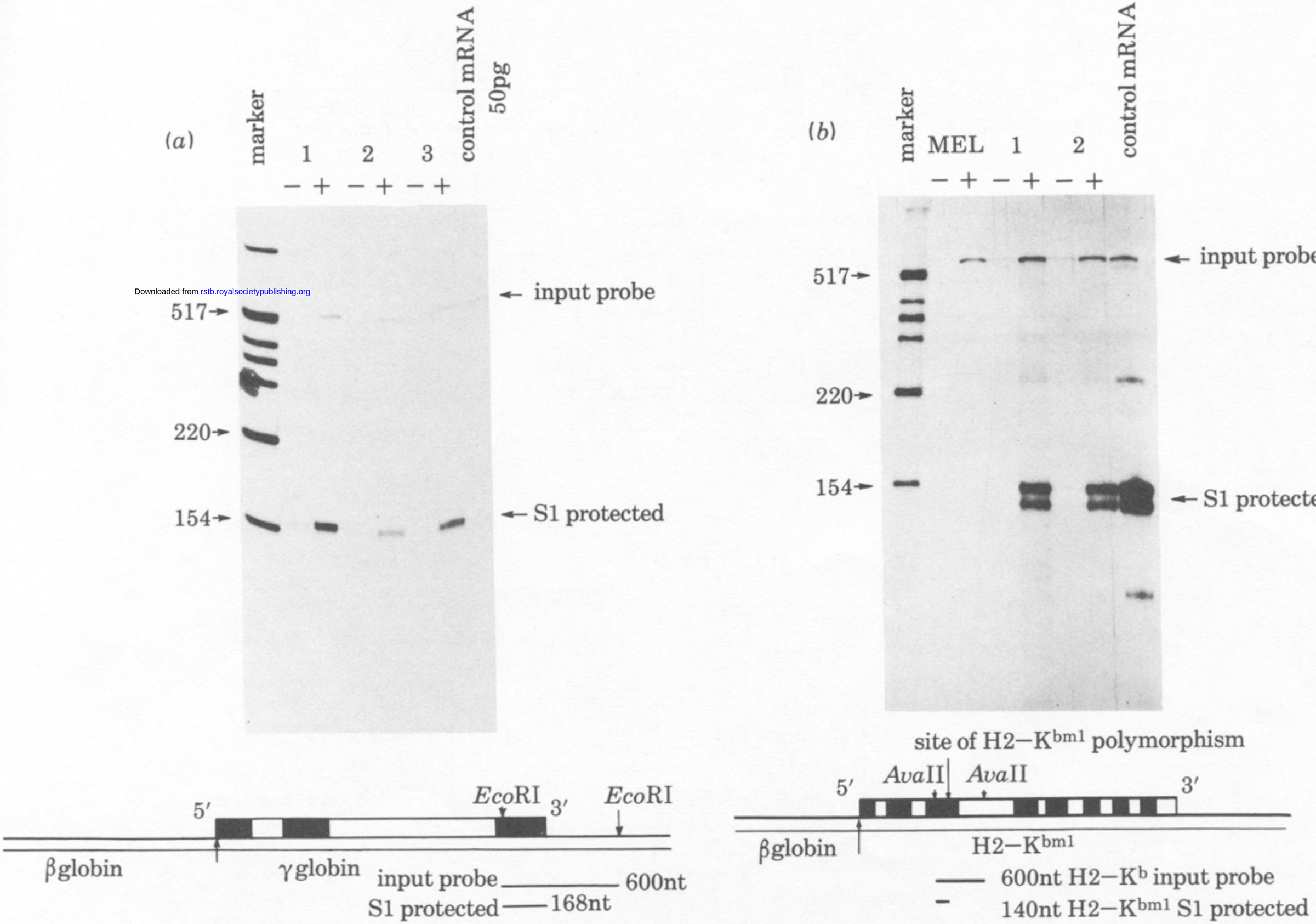
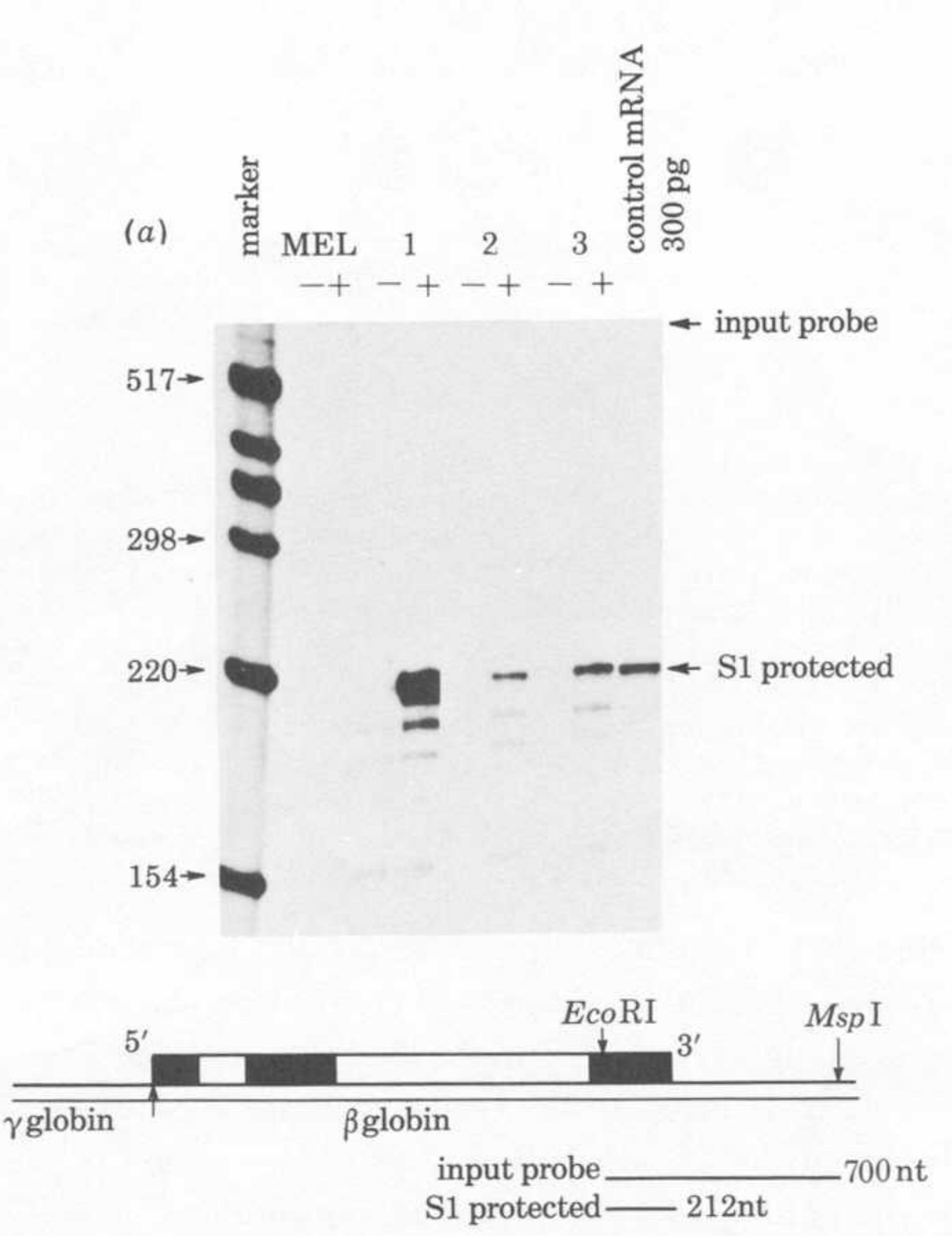


FIGURE 5. (*a*) S1 nuclease analysis of MEL cell transformants containing 5′β–3′γ hybrid globin genes. (*b*) S1 nuclease analysis of MEL cell transformants containing 5′β–3′H-2K^{bm1} globin genes. Control RNAs and methods were as described in figures 2 and 3.



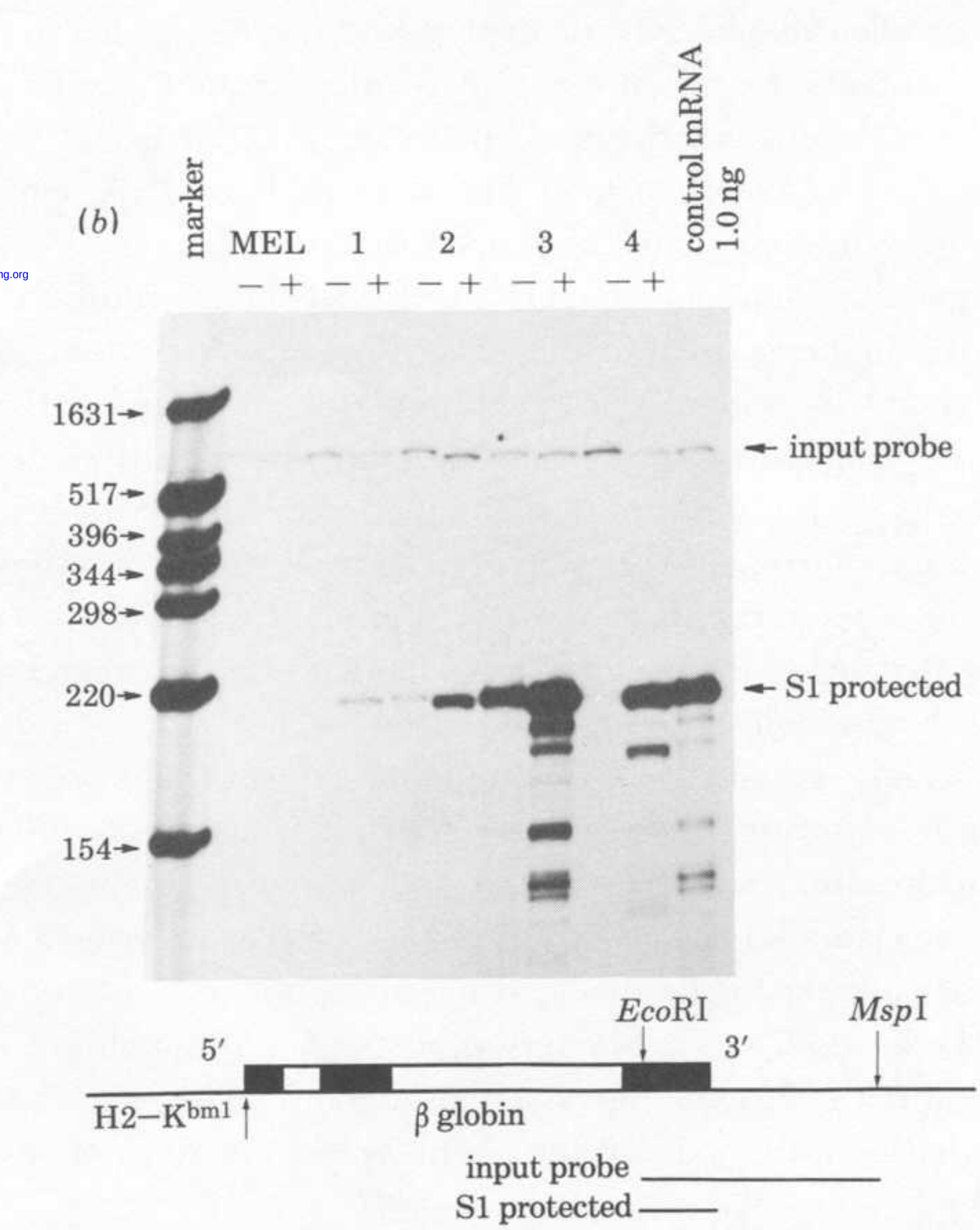


FIGURE 7. (*a*), (*b*) S1 nuclease analysis of the 5′γ–3′β and 5′H-2K^{bm1}–3′β hybrid genes respectively. Control RNA and methods were as described in the previous figures.

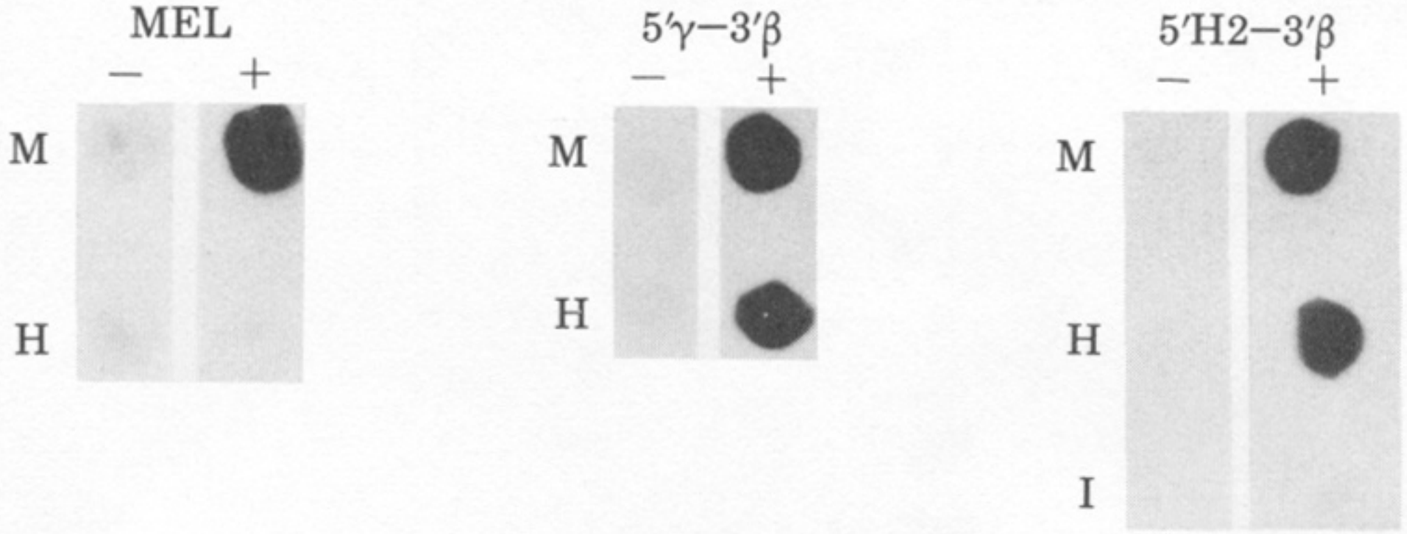


FIGURE 8. Nuclear run-off transcription of MEL cell transformants containing hybrid globin genes. Nuclei were isolated from non-induced (−) and induced (+) MEL cell transformants containing a 5′γ–3′β or a 5′H-2K^{bm1}–3′β hybrid gene. RNA was labelled with ^{32}P-UTP (300 μCi, 3000 Ci/mmol per 5×10^7 nuclei) as described by Groudine *et al.* (1984). Two μg of purified DNA fragments were bound to a 0.45 μm nitrocellulose filter, as described by Kafatos *et al* (1979), and hybridized with labelled nuclear transcript as described by Groudine *et al.* (1984). Each hybridization contained nuclear RNA from 10^7 cells which corresponded to 5×10^7 to 10×10^7 counts per minute for the non-induced cells and 2×10^7 to 5×10^7 for the induced cells. After hybridization, the filters were washed to a stringency of 0.3 × SSC. The human β-globin (H), mouse β-major globin (M) and human insulin (I) fragment probes were an *Eco*RI–*Bam*HI IVS fragment, a *Hin*III–*Pst*I IVS fragment and a 1.8 kilobases *Bam*HI fragment (Bell *et al.* 1982), respectively.

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