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α-Amanitin insensitive transcription of the human ε-globin gene

G.Kollias[+*], C.E.Sekeris[+] and F.G.Grosveld*

[+]National Hellenic Research Foundation, Biological Research Centre, Vas. Konstantinou 48, Athens, Greece, and *Laboratory of Gene Structure and Expression, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

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ABSTRACT

In vitro transcription was used to show that RNA polymerase III is responsible for the initiation of transcription at a position 200bp upstream from the ε-globin major cap site. High levels of -200 transcription interferes with the RNA polymerase II major cap site transcription. Using DNA mediated transient expression, the ratio of -200 to +1 transcription can be modulated by either the direction of replication or the presence of an enhancing element in the vector. We suggest that this heterogeneous usage of cap sites is not related to ε-globin gene transcription *in vivo*, but is instead the result of a combination of factors inherent to transient expression experiments.

INTRODUCTION

Recently a number of alternative initiation sites for transcription have been found in several viral and cellular genes (1-7). In one case, the amylase 1α gene, initiation heterogeneity was shown to correlate with tissue (4) and stage (8) specific expression of the gene. However, in most cases, such a correlation has not been rigorously established. For example, the majority of globin gene transcription initiates at the mRNA cap site in erythroid tissue, but there have been reports that at least the ε-, γand β-globin genes have other initiation sites in erythroid and other tissues (9, 10). In the case of the ε-globin gene, such sites have been mapped to four regions, -10 to -200, -850, -1430 and -4450bp from the major cap site (11). Most of these longer transcripts are capped and account for about 10-15% of the total ε-globin transcripts, both in an erythroleukaemic cell line and human embryonic erythroblasts (10). Approximately 5% of the total ε-globin transcripts in erythropoietic tissues originate from the -200 cap site alone (11). Preliminary evidence indicates that these longer transcripts follow the induction by hemin of the total ε-globin mRNA population in K562 cells (G. Kollias, unpublished). When non erythropoietic cell lines are transfected with recombinants containing the ε-globin gene together with the SV40 enhancer

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and/or the SV40 origin of replication, initiation of transcription from the -200 cap site occurs at relatively higher rates compared to the *in vivo* situation (12). Moreover, the -200 and the major cap site show a different response to the SV40 elements included in the expression vectors (12). The function of these upstream derived mRNAs with respect to the mechanism of ε-globin gene regulation is still unknown.

Analogous to *in vitro* experiments on the upstream derived transcripts of the human β- (13) and the mouse β-major (14) globin genes, we demonstrate in this paper that the ε-globin -200 cap site RNA is also transcribed *in vitro* by RNA polymerase III, while the +1 cap site RNA is transcribed by RNA polymerase II. We have studied the modulation of these two different promoters in transient assays using vectors containing the SV40 enhancer and/or the SV40 origin of replication. The results show that the direction of vector replication by itself can determine the usage of either the -200 cap site, or the major cap site. The results also suggest that transcriptional interference from the -200 cap site is a second mechanism that influences the expression of the +1 (major) cap site. Together, these two elements explain the heterogeneous response of the ε-globin gene +1 cap transcription to viral cis-acting controls.

MATERIALS AND METHODS

Transfection of cell cultures - RNA preparation - S1 mapping

K562 cells were grown in RPMI1640 medium supplemented with 10% fetal calf serum. Log phase cells were seeded at a concentration of 10^5 cells/ml. Cells were induced for four days in fresh medium containing 0.03mM hemin. HeLa cells were grown in α-MEM medium supplemented with 10% newborn calf serum. Calcium phosphate mediated DNA transfections were performed as described by Wigler *et al*. (15). 48 hrs after transfection, cells were harvested and total RNA was prepared by the method of Chirgwin *et al*. (16). S1 mapping analysis of the RNA (17, 18) was performed using radioactively labelled DNA probes as indicated in the figures.

In vitro transcription

Transcription extracts were prepared as described by Manley *et al* (19). Transcription assays were performed in 50μl containing 2.5μg DNA, 0.5mM ATP, 0.6mM CTP, 0.6mM GTP, 0.06mM UTP, 0.15mM EDTA, 1mM creatine phosphate and 10μCi [α-^{32}P]UTP (400 Ci/mmole). Reactions were incubated for 1 hr at 30°C, followed by incubation 10 min. at 30°C with an equal volume of proteinase K mix (400 μg/ml proteinase K, 10μg wheat germ tRNA, 25mM

EDTA, 100mM Tris-HCl pH. 7.5 and 2% SDS). The reaction mixture was then extracted with phenol-chloroform, ethanol precipitated, washed with 70% ethanol and dissolved in 7M Urea, 5mM Tris-borate 8.3, 1mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol. After heating at 100°C for 90 sec. RNA products were separated on a 5% polyacrylamide/urea gel (26).

RESULTS

RNA Polymerase III initiates transcription at the -200 cap site of the ε-globin gene

It is well known that eucaryotic cell-free extracts can direct transcription initiation events in various polymerase II or III DNA templates (20, 21). In order to determine which RNA polymerase is responsible for the transcription of the upstream derived ε-globin RNA molecules, we used *in vitro* transcription run-off assays with HeLa whole-cell extracts in the presence of α-amanitin. RNA was transcribed from two different DNA templates which contained either both the -200 and major cap site or only the latter. The size of the RNA's was then analyzed on 5% acrylamide-urea gels (Fig. 2).

When RNA was transcribed from a 650bp BamHI fragment (Fig. 1), which does not contain the -200 cap site, a band of approximately 475bp was observed as expected (Fig. 2c, lane 1). This band represents the RNA transcript which initiates at the major cap site of the ε-globin gene. Synthesis of this RNA species is inhibited by 2μg/ml α-amanitin indicating that it is transcribed by RNA polymerase II (Fig. 2C, lane 2). The bands of approximately 650bp in the same figure represent whole fragment transcripts always found in the *in vitro* assays.

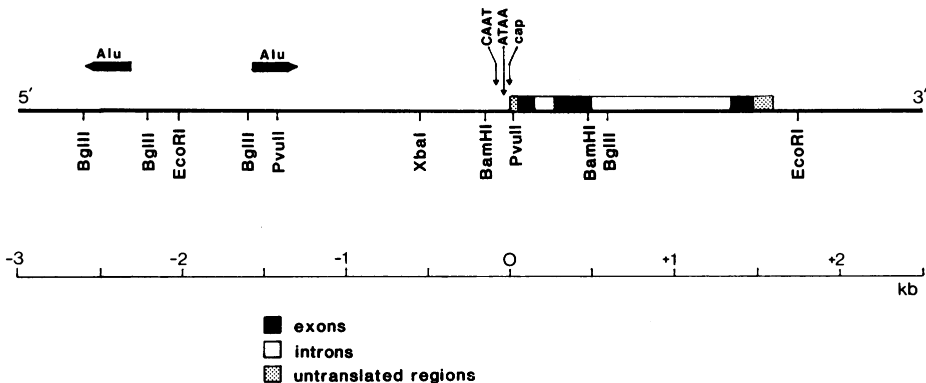


Fig. 1: Restriction map of the ε-globin gene and its flanking sequences.

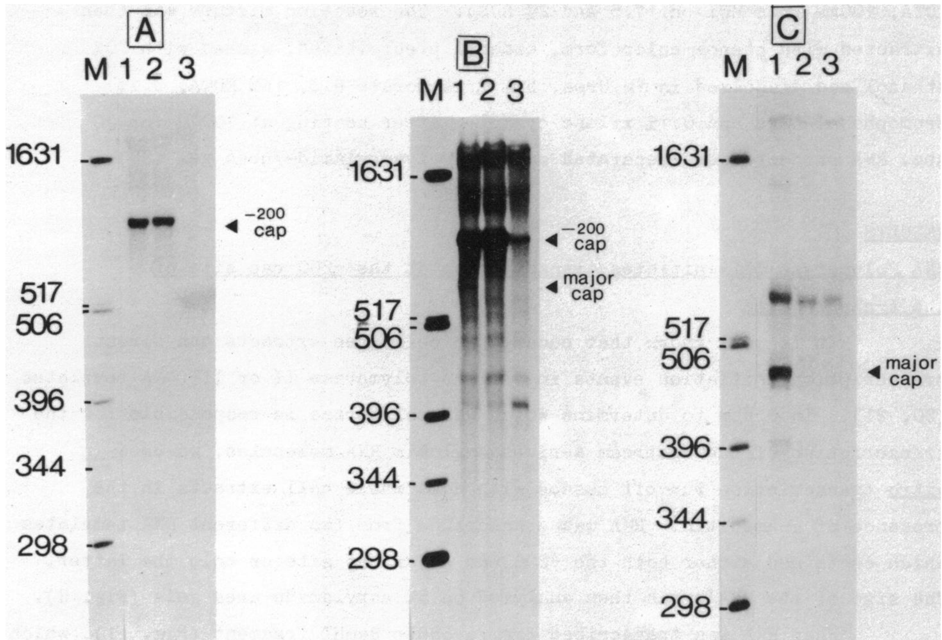


Fig. 2: Electrophoretic analysis of RNA synthesized on ε-globin templates. In vitro transcription assays were performed as described in Materials and Methods and the products were analyzed on a 5% polyacrylamide-urea gel.

(A): Transcription extracts were incubated with the 2.1kb BglII fragment of ε-globin gene (see Fig. 1A) containing both the -200 and the major cap site (lane 1) RNA synthesized without α-amanitin; (lane 2) 2μg/ml α-amanitin; (lane 3) 100μg/ml α-amanitin. (lane M) [γ-^{32}P]-ATP labelled pBR322 x Hinf I DNA fragments. Autoradiography was for 15hrs at -70°C with intensifying screen.

(B): As (A) but exposed for 5 days.

(C): Transcription extracts were incubated with the 0.6kb BamHI fragment of ε-globin gene (see Fig. 1A) containing the major cap site but not the -200 cap site (lane 1). RNA synthesized in the presence of no additives. (lane 2) 2μg/ml α-amanitin. (lane 3) 100μg/ml α-amanitin. (lane M) [γ-^{32}P]-ATP labelled pBR322 x HinfI DNA fragments. Autoradiography was for 15hrs at -70°, with intensifying screen.

Positions of RNA molecules with sizes corresponding to transcription initiation from the -200 cap site or the major cap site are indicated.

When RNA was transcribed from a 3190bp BglII fragment of the ε-globin gene (Fig. 1), the transcription pattern is quite different. This fragment contains both the -200 cap site and the major cap site, together with 1.6kb of 5' flanking sequences. A prominent band of approximately 800bp was detected which represents the RNA transcript originating from the -200 cap site (Fig.

2A, lane 1). Synthesis of this RNA is not blocked by 2μg/ml α-amanitin (Fig. 2A, lane 2), but is inhibited by 100 μg/ml α-amanitin (Fig. 2A, lane 3) indicating that it is transcribed by an α-amanitin insensitive RNA polymerase. In a longer exposure of the same gel a very faint band of approximately 600bp (Fig. 2B, lane 1) corresponding to the +1 cap site is visible. As expected, this band is sensitive to 2μg/ml α-amanitin (lane 2). Other minor bands of no obvious significance are also visible even after treatment with 100μg/ml α-amanitin.

It is interesting to note that active transcription from the -200 cap site lowers the efficiency of transcription from the major cap site (Fig 2B versus Fig. 2C) which suggests that a mechanism of transcriptional interference might also act in vivo (see below) to regulate the quantitative representation of the two different RNA populations in the total ε-globin RNA.

Correct processing of the -200 cap site derived ε-globin

To demonstrate that the ε-globin RNA transcripts originating from the -200 cap site undergo correct processing, we carried out an S1 blot experiment (Fig. 3) and confirmed the results by S1 mapping analysis (data not shown). Total RNA was isolated from uninduced and induced K562 cells and from HeLa cells transiently transformed with an ε-pBSV construct (see below). In the pBSV vector both the SV40 enhancer and the SV40 origin of replication are present. This RNA was hybridized with the 3.7kb EcoRI fragment containing the complete ε-globin gene and upstream region. The DNA-RNA was digested with S1 endonuclease and electrophoresed on a 1.5% alkaline agarose gel. The DNA bands were transferred to a nitrocellulose filter and hybridized to a ^{32}P-nick translated 3.7kb EcoRI fragment. In the case of K562 RNA (Fig. 3, lanes 2, 3), a single band of 147 and a double band of 221 and 248 nucleotides were observed, corresponding to the exact size of the three exons of the ε-globin gene. However, in the case of the transiently expressed ε-globin RNA from the HeLa cell experiment, an additional prominent band of 360 nucleotides was observed. This corresponds to the distance from the -200 cap site to the first exon/intron junction, indicating ε-globin RNA molecules which are transcribed from the -200 cap site undergo correct processing at the same splice junctions as the +1 cap site ε-globin mRNA. Moreover, the -200 cap site transcript is polyadenylated and shows the same levels of inducibility by hemin (2-3 fold) as the major cap site in K562 cells (data not shown). Comparison of the relative activities between the -200 and the major cap site in Fig. 3, lane 1, suggests that transcription interference of the -200 cap site transcription with the major cap site transcription also plays a

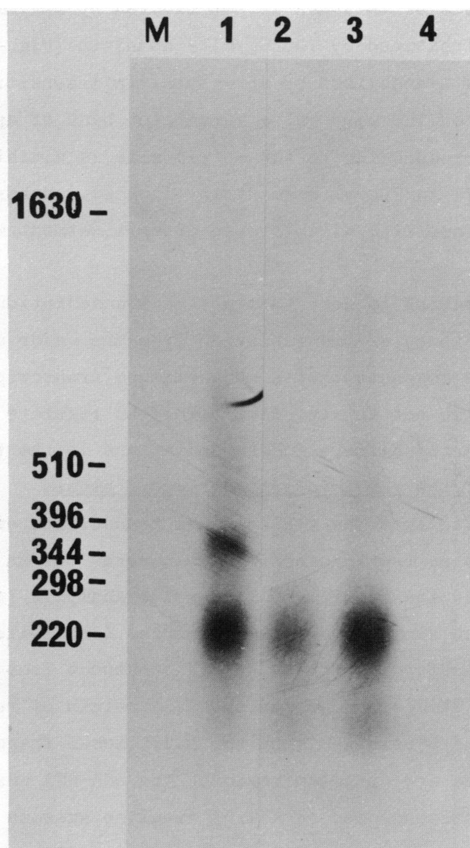


Fig. 3: S1 blot analysis of ε-globin mRNA species using the 3.7kb EcoRI fragment of ε-globin gene:

S1 hybridizations were performed as described in Materials and Methods using (1) total RNA from HeLa cells transfected with the 3.7εpBSV (5'->3') vector; (2) total uninduced K562 RNA and (3) total induced K562 RNA and the 3.7kb EcoRI fragment of ε-globin gene. The S1 products were electrophoresed on a 2% alkaline agarose gel (Favoloro et al, 1980), transferred to nitrocellulose filters and hybridized with a ^{32}P-nick-translated 3.7kb EcoRI fragment of ε-globin gene. (lane M) [γ-^{32}P]-ATP labelled pBR322 x HindfII DNA fragments. Autoradiography was for 5 hours at -70°C with intensifying screen.

role in the HeLa cells, since the total sum of the relative densities of the three exon bands is equal in the HeLa and K562 cells.

Effect of vector replication on the transcription of ε-globin gene in HeLa cells.

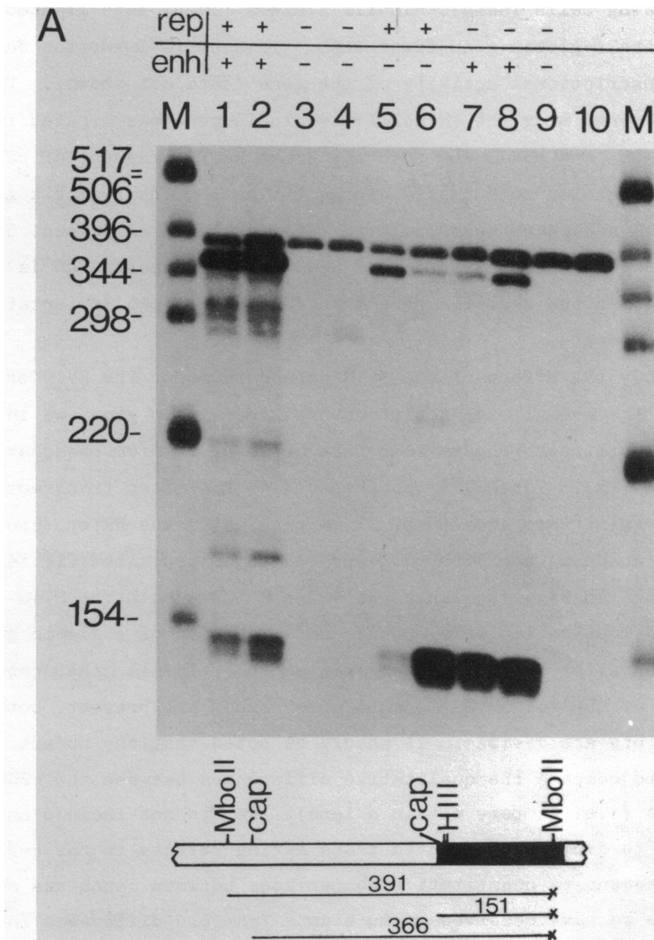
The 3.7kb EcoRI fragment containing the ε-globin gene was cloned into

the SV40 vectors pBSV (22) pSVOD (23) and pSV2 (24). pBSV contains the 3.3kb EcoRI-BamHI fragment of pBR328 linked to the 4.7kb EcoRI-BamHI fragment of SV40. Thus, this vector contains the SV40 enhancer and origin of replication sequences and can synthesize large T antigen required for replication. pSVOD contains the 311bp EcoRII fragment of SV40 inserted into the EcoRI site of essentially pBR327 with a deletion between HindIII site in pBR327 and the HindIII site in SV40. This vector, therefore, contains the SV40 origin of replication, but lacks the SV40 enhancer sequences. pSV2 contains only the SV40 enhancer and the SV40 promoter sequences linked to the chloramphenicol acetyl transferase (CAT) gene, together with pBR327. The SV40 origin of replication is not included in this plasmid. To be able to use the same constructs for transient expression experiments in ε-globin expressing (K562) and non expressing cells (HeLa), HindIII linkers (20bp) were ligated into the PvuII site of the ε-globin gene first exon. This small insertion does not affect the transcriptional activity of the gene (data not shown). The 3.7kb EcoRI fragment containing the (HindIII+) ε-globin gene was ligated into the EcoRI site of pBSV and pSVOD and into the 2.8kb EcoRI fragment of pSV2. Recombinants containing both possible orientations of the ε-globin gene relative to SV40 sequences were isolated (Fig. 4B). In all cases, 5'->3' orientation indicates that direction of SV40 early transcription is in the same orientation as the ε-globin gene transcription; 3'->5' orientation indicates the opposite.

To study the effect of the SV40 enhancer and/or the SV40 origin of replication on the overall transcription of the ε-globin gene, we introduced all of the ε-globin recombinants into HeLa cells by calcium phosphate coprecipitation (15). Total RNA was prepared 48 hrs after transfection and 5' ε-globin RNA termini were located by S1 mapping using the 391bp (HindIII+) MboII fragment spanning both the -200 and the major cap site (Fig. 4A).

As shown in Fig. 4A, lanes, 3, 4 and 9, when both the SV40 enhancement and replication were absent, no expression of ε-globin gene could be detected even after long exposures (not shown). In all other cases where both or either of the two SV40 elements under study are present, both -200 and +1 cap transcripts are visible. It should be noted that the object of this experiment is to compare the qualitative differences between the -200 and +1 cap-transcripts (i.e. compare within a lane). We did not include an internal marker plasmid to avoid any unwanted trans-acting effects on the ε-globin plasmid. Consequently, quantitative comparisons between lanes are not accurate, since we have observed as much as a two-fold difference in

transformation efficiencies between experiments. The presence of the SV40 enhancer in the vector (lanes 7 and 8) gives an increase in the number of transcripts originating from both the -200 and +1 cap sites. The difference between the -200 signal from the two gene orientations (lanes 7 and 8) is not consistent when several experiments are compared with each other. However, an orientation effect is very reproducible when the vector is replicated by the addition of an SV40 origin (lanes 5 and 6). The pattern of transcription from the two cap sites seems to be inverted with respect to the orientation of the replication fork. Activation of the -200 cap site is highest when the transcribed strand of the ε-globin gene corresponds to the discontinuously synthesized strand. In the opposite orientation, the major cap site is



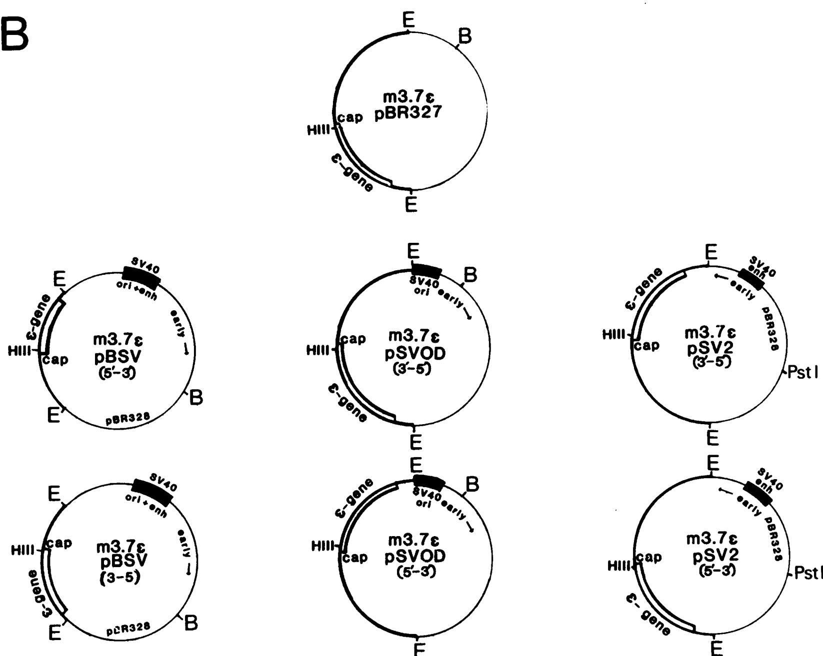


Fig. 4A: Effect of cis-acting SV40 elements on the transcription of ε-globin gene in short term transformants.

HeLa cells were transfected with 10μg of (1) m3.7εpBSV (5'->3'), (2) m3.7εpBSV (3'->5'), (3) m3.7εpSVOD (5'->3'), (4) m3.7εpSVOD (3'->5'), (5) m3.7εpSVOD (5'->3') + 10μg pBSV, (6) m3.7εpSVOD (3'->5') + 10μg pBSV, (7) m3.7εpSV2 (5'->3'), (8) m3.7εpSV2 (3'->5') and (9) m3.7εpBR327. In all cases except (5) and (6) the cells were cotransfected with 10μg of salmon sperm DNA. Total RNA was prepared 48 hrs later and ε-globin RNA 5' termini were mapped by S1 nuclease using a 5' labelled MboII-MboII probe. Total HeLa cell RNA (lane 10) was used as a negative control. Lane M contains pBR322 x HinfI fragments. Autoradiography was for 45 hrs at -70°C with intensifying screen. The presence of the SV40 enhancer and the function of the SV40 origin of replication on the transfection vectors is indicated on the upper part of the figure. Expected sizes for the -200 or the major cap site protection of the probe is shown at the bottom of the figure.

Fig. 4B: Construction of transfection vectors:

The 3.7kb EcoRI fragment of the human ε-globin gene was inserted into the EcoRI site of vectors (pBSV (22), pSVOD (23) and pSV2 (24). pBSV contains both the SV40 origin of replication and the SV40 enhancer, pSVOD contains only the SV40 origin of replication and pSV2 contains only the SV40 enhancer. The orientation of ε-globin transcription and SV40 early transcription is indicated.

activated. This result, therefore, supports the model by Smithies (25) which proposes differential activity of replication as a mechanism to control gene activation. Interestingly, when replication takes place in the presence of an enhancer (lanes 1 and 2), the stimulatory effect on the -200 cap site

transcription appears to be "additive" and leads to such an effective -200 cap transcription that it interferes with the downstream +1 cap transcription to give a level lower than that found in the presence of only an enhancer.

DISCUSSION

In recent years information has accumulated concerning alternative transcription initiation sites for the human β-related globin genes (9, 12). The most studied case has been that of the human embryonic ε-globin gene, in which transcription from a site -200bp upstream from the major cap site accounts for approximately 5% of the total ε-globin gene expression in human embryos and in cultured cell lines (10). Quite surprisingly, the -200 cap site (11) is used for unknown reasons to a greater extent (up to 80% of the total ε-globin transcripts) in short term transformed cell lines (12) and under the control of cis-acting SV40 elements.

It has recently been reported (13, 14) that the β-globin upstream-derived RNAs are transcribed in vitro by RNA pol III and that the sequences flanking the proposed initiation sites closely resemble classical pol III promoters. No information was available as to which polymerase transcribes the upstream derived ε-globin species. We show that an α-amanitin insensitive polymerase produces RNAs of the same size as those that originate from a site -200bp upstream from the major ε-globin cap site in vivo (Fig. 2). Although RNA pol III would be the best candidate for such properties, a comparison of the ε-globin sequences around the alternative initiation site with pol III promoter consensus sequences (27) failed to reveal a strong homology. The longer transcripts behave similarly to the major cap site derived ones with regard to splicing (Fig. 3) and inducibility by hemin (not shown). At this stage we can only speculate on the role of these RNA molecules in vivo, either in general cellular mechanisms or more specifically in the regulation of globin gene expression. There is a possibility that these longer pol III transcripts could play a trans-acting regulatory role, either as an RNA molecule, or by the production of a polypeptide. There are indeed two possible AUG codons between the two alternative cap sites, translation from which could give rise to polypeptides with 41 and 38 amino acids. Alternatively, the upstream transcript could have a cis regulatory role on the transcription of the ε-globin gene. For example, the results in Fig. 2 suggest that interference could take place by the -200 cap on the +1 cap transcription. However, since a high level of -200 cap transcripts or a changing ratio of -200 to +1 cap transcripts is not observed in human tissues

(10), _in vivo_ interference appears unlikely.

When plasmids containing the ε-globin gene, but lacking both viral elements, are introduced into HeLa cells (3.7εpBR327 - Fig. 4A, lane 9) transcription from either the -200 cap site or the major cap site is undetectable (even after long exposure times). The same result is obtained when HeLa cells are transfected with the 3.7εpSVOD construct alone (Fig. 4A, lanes 3, 4). In the latter case there is no T-antigen for the replication from the SV40 origin and the vector remains inactive. At present we do not know why this result is different from previous observations which showed a 50-fold greater transcription initiation from the -200 cap site over that from the major cap site (12), although it should be pointed out that a different cell line was used.

In all other cases when plasmids containing the ε-globin gene, together with the SV40 enhancer and/or the origin of replication were used, initiation of transcription occurred at both the -200 cap site and the major cap site. It has been shown that the SV40 enhancer increases RNA pol II density within the linked gene (28, 29) which would explain the high level of transcription from the ε-globin major cap site in relation to the -200 cap site in the 3.7εpSV2 vector (enhancer only, Fig. 4A, lanes 7, 8). The increased density of RNA pol II molecules at the major cap site promoter may not allow efficient transcription of the RNA pol III transcribed -200 cap site.

The most interesting result was obtained when we used the ε-globin pSVOD consruct cotransfected with the pBSV vector. The result clearly shows that in the orientation in which the direction of transcription of the ε-globin gene is colinear with the continuously synthesized strand of replication, initiation of transcription from the major cap site is much more active than from the -200 cap site (Fig. 4A, lane 6). The pattern closely resembles the _in vivo_ situation in erythropoietic cells. In the opposite orientation of the replication fork (Fig. 4A, lane 5), the -200 cap site seems to be stimulated while the major site shows very little activity (which could be due to the transcriptional interference from the active -200 cap site). It seems, therefore, that the asymmetry of the replication event is reflected in an asymmetrical pattern of ε-globin gene transcription in relation to the -200 and the major cap site. Limited support for replication influencing upstream transcription has been obtained by studies in adenovirus (30) and SV40 (31).

In summary, data presented here and previous transient expression experiments (12) demonstrate heterogeneous regulation of the ε-globin gene

-200 and major cap site transcription in relation to the SV40 elements used. We would suggest that extreme care should be taken with the interpretation of transient expression experiments when multiple factors are changed by the introduction of an enhancer or an origin of replication. In contrast to the previous report (12) we would consider it most likely that the heterogeneous -200 cap/+1 cap expression is the result of in vitro manipulation (vectors, cell lines etc.) in transient expression experiments. The wider significance of our data for the normal regulation of the human ε-globin gene transcription in the yolk sac in vivo, are implied from the demonstration that transcriptional interference (negative) or the direction of replication could play a regulatory role.

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REFERENCES

1. Grez, M., Land, H., Giesecke, K., Schütz, G., Jung, A. and Sippel, A. E. (1981) Cell, 25, 743-752.
2. Gerlinger, P., Krust, A., Le Meur, M., Perrin, F., Cochet, M., Gannon, F., Dupret, D. and Chambon, P. (1982) J. Mol. Biol. 162, 345-364.
3. Lai, E. C., Roop, D. R., Tsai, M., Woo, S. L. C. and O'Malley, B. W. (1982) Nucl. Acids Res., 10, 5553-5567.
4. Young, R. A., Hagenbüchle, O. and Schibler, V. (1981) Cell, 23, 451-458.
5. Cowie, A., Tyndall, C. and Kamen, R. (1981) Nucl. Acids Res., 9, 6305-6322.
6. Haegeman, G. and Fiers, W. (1980) J. Virol., 35, 955-961.
7. Ghosh, P. K., Reddy, V. B., Swinscoe, J., Lebowitz, P. and Weissman, S. M. (1978) J. Mol. Biol., 126, 813-846.
8. Shaw, P., Sordat, B. and Schibler, U. (1985) Cell, 40, 907-912.
9. Grindlay, G. J., Lanyon, W. G., Allan, M. and Paul. J. (1984) Nucl. Acids Res., 18, 1809-1820.
10. Allan, M., Grindley, G. J., Stefani, L. and Paul, J. (1982) Nucl. Acids Res., 10, 5133-5147.
11. Allan, M., Lanyon, W. G. and Paul, J. (1983) Cell, 35, 187-197.
12. Allan, M., Zhu, J., Montague, P. and Paul, J. (1984) Cell, 38, 399-407.
13. Carlson, D. P. and Ross, J. (1983) Cell, 34, 857-864.
14. Carlson, D. P. and Ross, J. (1984) Proc. Natl. Acad. Sci. U.S.A., 81, 7782-7786.
15. Wigler, M., Sweet, R., Sim, G. K., Wold, B., Pellicer, A.,

Lacy, E., Maniatis, T., Silverstein, S. and Axel, R. (1979) Cell, 16, 777-785.
16. Chirgwin, J. M., Pizybyla, A. E., MacDonald, R. J. and Putter, W. (1979) Biochemistry, 18, 5294-5299.
17. Berk, A. J. and Sharp, P. A. (1977) Cell, 12, 721-732.
18. Weaver, R. F. and Weissmann, C. (1979) Nucl. Acids Res., 6, 1175-1193.
19. Manley, J. L., Fire, A., Cano, A., Sharp, P. A. and Gefter, M. L. (1980) Proc. Natl. Acad. Sci. U.S.A., 77, 3855-3859.
20. Manley, J. L., Fire, A., Cano, A., Sharp, P. A. and Gefter, M. L. (1980) Proc. Natl. Acad. Sci. U.S.A., 77, 3855-3859.
21. Manley, J. L. and Colozzo, M. T. (1982) Nature, 300, 376-379.
22. Grosveld, G. C., deBoer, E., Shewmaker, C. K. and Flavell, R. A. (1982) Nature, 295, 120-126.
23. Mellon, P., Parter, V., Gluzman, Y. and Maniatis, T. (1981) Cell, 27, 279-288.
24. Gorman, C., Moffat, L. and Howard, B. (1982) Mol. Cell. Biol., 2, 1044-1051.
25. Smithies, O. (1982) J. Cell Phys. Suppl. 1, 137-143.
26. Maxam, A. and Gilbert, W. (1980) Meth. Enzymol., 65, 499-560.
27. Sharp, S., DeFranco, D., Dingerman, T., Farrell, P. and Söll, D. (1981) Proc. Natl. Acad. Sci. U.S.A., 78, 6657-6661.
28. Weber, F. and Schaffner, W. (1985) Nature, 315, 75-77.
29. Treisman, R. and Maniatis, T. (1985) Nature, 315, 72-75.
30. Osborne, T. F. and Berk, A. J. (1983) J. Virol., 45, 594-599.
31. Ghosh, P. K. and Lebowitz, P. (1981) J. Virol., 40, 224-240.