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Highly efficient β globin transcription in the absence of both a viral enhancer and erythroid factors

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Summary

We have studied the transcription of the *Xenopus* major adult β globin gene in microinjected *Xenopus* oocytes at various levels of injected template, with or without the SV40 enhancer. We find that enhancer-independent transcription is highly efficient, being only two orders of magnitude below the calculated *in vivo* rate. Linkage to the SV40 enhancer has very little stimulatory effect. We have also tested the effect of replication on transcription in the oocyte system where replication was induced by progesterone treatment followed by prick activation. We found that the presence of replicated templates did not stimulate expression of the *Xenopus* β globin gene either in the presence or absence of the SV40 enhancer. In addition, we found that specificity of transcription, in terms of initiation at the cap site *versus* initiation at cryptic promoters upstream of the cap site, was dramatically improved by the injection of higher numbers of β globin templates, by oocyte maturation and activation or by the presence of the SV40 enhancer.

Key words: β globin, replication, SV40 enhancer and early promoter, transcription factors, *Xenopus* oocytes.

Introduction

Globin genes are extreme examples of genes with a tissue-specific expression profile, transcripts only being detectable in erythroid cells *in vivo*. The mechanisms by which expression is restricted in this way are not yet elucidated but a number of observations have suggested the involvement of tissue-specific factors interacting with genetic elements near the genes (Emerson & Felsenfeld, 1984; Townes *et al.* 1985; Chada *et al.* 1985; Kollias, Wrighton, Hurst & Grosveld, 1986; Hesse, Nickol, Lieber & Felsenfeld, 1986; Choi & Engel, 1986; Rutherford & Nienhuis, 1987; Emerson, Nickol, Jackson & Felsenfeld, 1987; Kollias, Hurst, deBoer & Grosveld, 1987).

When transfected into nonerythroid cells, β globin genes are expressed only when linked to viral enhancer sequences and, in general, this enhancer-driven expression is faithful in terms of initiation of transcription from the authentic cap site alone (see Serfling, Jasin & Schaffner, 1985 for review). In the specific case of the *Xenopus* β globin gene, transcription in transfected HeLa cells has been shown to be not only enhancer dependent but also stimulated by DNA replication (Enver, Brewer & Patient, 1987).

Although *Xenopus laevis* oocytes are now well established as an efficient and accurate expression system for genes transcribed by RNA polymerase III, they are generally reported to be a poor system for the expression of polymerase II genes and, in particular, for those genes whose expression is tissue specific *in vivo* (for review see Gurdon & Melton, 1981). With the exception of the human zeta globin gene (Proudfoot, Rutherford & Partington, 1984) and the chicken histone H5 gene (Wigley, Strum & Wells, 1985), tissue-specific genes, when injected into *Xenopus* oocytes, are inaccurately expressed, resulting in low levels of transcripts initiated from multiple sites other than the cap site (Wickens, Woo, O'Malley & Gurdon, 1980; Trendelenberg, Mathis & Oudet, 1980; Rungger, Matthias & Huber, 1981; Bendig & Williams, 1984*a*; Partington, Yarwood & Proudfoot, 1984). Furthermore, for the *Xenopus* β globin gene, fidelity of transcription has been shown to vary dramatically between oocytes from different individuals (Bendig & Williams, 1984*b*).

We have investigated the expression of the *Xenopus* β globin gene when injected into *Xenopus* oocytes under a variety of conditions including the presence or absence of enhancer sequences, the induction of oocyte maturation and DNA replication, and the varying of injected template levels over a range not limiting for chromatin assembly in the oocyte (Gargiulo, Razvi & Worcel, 1984). Our results show that, even without linkage to the SV40 enhancer and in the absence of erythroid-specific factors, the β globin gene is a highly efficient template for transcription.

Materials and methods

Preparation of DNA samples for injection

Plasmid DNA purified through two caesium chloride gradients was precipitated with ethanol and resuspended in injection buffer (80 mM-NaCl, 50 mM-Tris–HCl pH 7·5) at concentrations of 100 μg ml^{-1}, 500 μg ml^{-1} or 1 mg ml^{-1}. For the titration experiments, 10 or 20 nl DNA at the above concentrations were injected into oocyte nuclei resulting in the deposition of 1, 5 or 20 ng DNA per oocyte. For the competition experiment (Fig. 3), 1 ng of the β globin containing plasmid pXGβ1RH (see Fig. 1) was co-injected with increasing amounts of the plasmid pSVENH, which consists of the 250 bp enhancer-containing cassette from SV40 (see Fig. 1) cloned into pUC18. The amount of DNA injected in this experiment was held constant at 20 ng by the addition of carrier pUC18 DNA.

Oocyte injection and nucleic acid extraction

Isolation and injection of stage VI oocytes (Dumont, 1972) was as described (Bendig & Williams, 1984*a*). Briefly, oocytes were treated with collagenase to remove ovarian follicular material and centrifuged to bring the nucleus to the cell surface. After injection, oocytes were incubated for 24 h at 18°C in modified Barth's saline. 15 healthy oocytes were selected for analysis and snap frozen in liquid nitrogen and stored at −70°C. Total nucleic acid was extracted from the 15 pooled oocytes as described (Gurdon & Wickens, 1983). Oocytes were homogenized in 0·3 M-NaCl, 2 % SDS, 50 mM-Tris–HCl pH 7·5, 1 mM-EDTA, 1 mg ml^{-1} proteinase K and extracted with phenol, chloroform, isoamyl alcohol 25:24:1. The phenol layer was back-extracted with the same buffer minus the proteinase K. Nucleic acid from the combined aqueous phases was precipitated with ethanol, washed with 70 % ethanol and resuspended in 1 mM-EDTA treated with diethyl pyrocarbonate.

Primer extension analysis

Primer extension analysis of steady-state RNA synthesized in injected oocytes was performed as described (Williams & Mason, 1985). The primer was an oligonucleotide 17 ntds long complementary to sequences from +88 to +104 in the first exon of the *Xenopus* β globin gene. The oligonucleotide was labelled at its 5′ end by polynucleotide kinase and should yield an extension product of 104 ntds with mRNA specifically initiated at the cap site. Routinely, we obtain a doublet of 104 and 107 ntds due to micro-heterogeneity of initiation at the cap site. The enhancer-containing construct should give rise to extension products of 116 and 119 ntds due to a 12 bp insertion in the first exon. Labelled primer (0·1 pmoles of 5′ ends) was hybridized to an aliquot of total RNA representing two to five injected oocytes in 0·4 M-NaCl, 10 mM-Pipes pH 6·4 at 52°C for 2–2·5 h in a final volume of 10 μl. 90 μl of primer extension mix (55 mM-Tris–HCl pH 8·2, 11 mM-DTT, 6·7 mM-$MgCl_2$, 27·8 μg ml^{-1} actinomycin D, 0·55 mM-dNTPs, 10 units reverse transcriptase) was added to each sample and primer extension was performed for 1 h at 42°C. After ethanol precipitation, samples were analysed by electrophoresis on a 6 % denaturing polyacrylamide gel.

Southern injection controls

Samples representing total nucleic acid extracted from a single injected oocyte were run on a 1 % agarose gel without ethidium bromide in a Tris–phosphate buffer (0·03 M-Tris–HCl pH 7·3, 0·036 M-NaH_2PO_4, 0·1 mM-EDTA) along with 5 ng of supercoiled, nicked circular and linear plasmid markers. Samples were blotted onto nitrocellulose and probed with nick-translated pAT153 vector DNA (Rigby, Dieckmann, Rhodes & Berg, 1977), or with a ^{32}P-labelled *Bcl*I–*Bgl*II fragment internal to the *Xenopus* β globin gene prepared by the mixed oligonucleotide priming technique (Feinberg & Vogelstein, 1984).

Assays for CAT expression

CAT assays were performed as described (Gorman, Moffat & Howard, 1982) except that only 1 μCi of [^{14}C]chloramphenicol (Amersham International) was used per assay. 30–40 injected oocytes were homogenized in 0·25 M-Tris–HCl pH 7·8 using 10 μl buffer per oocyte. The homogenate was centrifuged for 5 min in a microfuge at 4°C, and 20 μl of the supernatant was assayed for CAT activity. Quantitation by scintillation counting of excised labelled products was performed as described (Gorman *et al.* 1982).

Oocyte maturation and prick activation

Maturation and activation of injected oocytes was as described (Harland & Laskey, 1980). Following nuclear injection, oocytes were immediately transferred to modified Barth's saline containing 1 μg ml^{-1} progesterone (Sigma) and incubated for 18 h at 18°C. Oocytes which had undergone germinal vesicle breakdown as a result of the hormone treatment were selected. Such oocytes are readily identified since they develop a diffuse white spot in the pigmented area of the oocyte (Gurdon, 1967). Selected oocytes were activated by a needle prick and were collected for analysis after a further 6 h incubation at 18°C.

Assay for the occurrence of DNA replication in activated oocytes

Samples representing total nucleic acid extracted from a single injected activated oocyte were digested with *Mbo*I (a restriction enzyme which cuts only doubly replicated demethylated DNA) and run on a 1 % agarose gel without ethidium bromide in parallel with the same undigested samples. Control samples of DNA extracted from injected oocytes and digested with *Sau*3AI, an isoschizomer of *Mbo*I which cuts both methylated and demethylated DNA,

were also run producing a characteristic pattern of bands in a limit digest. Samples were blotted onto nitrocellulose and probed with nick-translated pAT153 vector DNA. The occurrence of replication was assessed by a reduction in supercoiled monomer DNA and the concomitant appearance of *MboI* digestion products in the digested samples compared to the undigested controls.

Results

The *Xenopus* β1 globin constructs used for injection are shown in Fig. 1. pXGβ1 contains the β globin gene only, whereas pXGβ1RH-enh-B contains the β globin gene linked to the SV40 enhancer. Each of these constructs was injected into *Xenopus* oocytes over a range from 1 to 20 ng per oocyte. To control for injection success, total nucleic acid was extracted from pooled, injected oocytes after a 24 h incubation at 18°C and a sample representing the amount extracted from a single oocyte was run on a 1% agarose gel, along with supercoiled, nicked circular and linear markers, blotted onto nitrocellulose and probed with nick-translated pAT153. In this way, we ascertained that (a) a proper titration of injected DNA had been achieved and (b) the DNA recovered was principally supercoiled, the topological form known to be the template for RNA synthesis by polymerase II in injected oocytes (Harland, Weintraub & McKnight, 1983). Furthermore, supercoiled DNA is an accurate measure of template deposited in the nucleus, since DNA injected into oocyte cytoplasm becomes progressively nicked, linearized and finally degraded (Gurdon & Melton, 1981).

Transcription of the Xenopus *β globin gene is highly efficient in* Xenopus *oocytes*

When the *Xenopus* β globin gene in the plasmid pXGβ1 was injected into *Xenopus* oocytes, active but inaccurate transcription was observed as measured by a primer extension assay of stable RNA. As well as transcripts initiating at the cap site, many longer transcripts representing initiations upstream of the cap site and extending back into vector sequences were detected (Fig. 2A, lanes 1 and 2). This phenomenon has been reported previously for the *Xenopus* β1 globin gene, and the upstream transcripts have been shown to be polymerase II dependent since they are sensitive to low levels of α-amanitin (Bendig & Williams, 1984*a*). Furthermore, the ratio of cap site to upstream starts varies among individual frogs

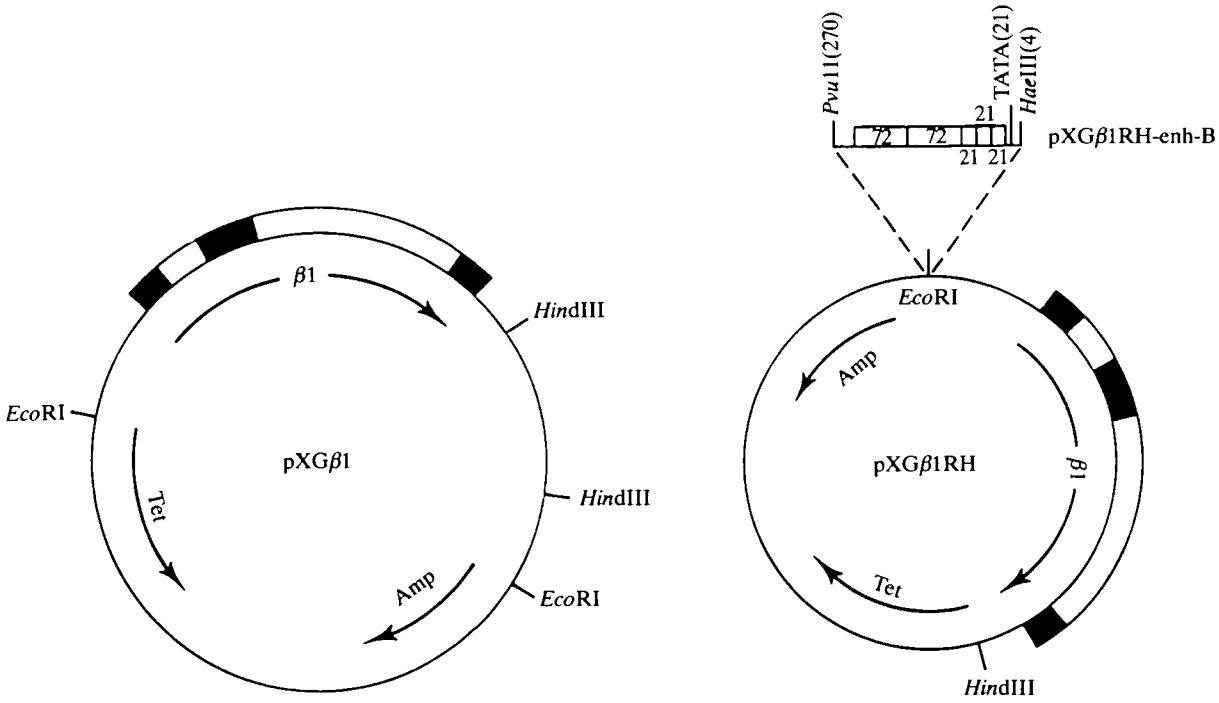

Fig. 1. Constructs used for microinjection. pXGβ1 (Patient, Harris, Walmsley & Williams, 1983) contains a 3·5 kb *Eco*RI *X. laevis* β1 globin gene fragment cloned into the *Eco*RI site of pAT153. pXGβ1RH contains a 2·36 kb *Eco*RI/*Hin*dIII *X. laevis* β1 globin gene containing fragment cloned into the *Eco*RI and *Hin*dIII sites of pAT153. In addition, a 12 bp (in phase) insertion was made into the *Bcl*I site in the first exon of the β1 gene using two selfligated *Bam*HI linkers which were then cleaved to reveal 4 bp sticky ends complementary to those produced by *Bcl*I cleavage (Bendig & Williams, 1984*b*). pXGβ1RH was cleaved at its unique *Eco*RI site to allow the insertion of an *Eco*RI-linkered, 250 bp enhancer 'cassette' to produce pXGβ1RH-enh-B (Enver *et al.* 1987).

(Bendig & Williams, 1984*a*). We show here that, within one individual, the ratio of cap site to upstream starts also varies over a concentration range of 1–20 ng of injected plasmid. As the levels of injected DNA increased, cap site initiations were augmented and upstream initiations reduced (Fig. 2A, lanes 1–3). In oocytes from four other individuals, the same effect was observed except that the template level required for maximal cap site signal and minimal upstream initiations was 1 ng for two of the frogs (M. Leonard, unpublished observations) and 80 ng for the other two (data not shown). The most plausible interpretation of these results is that the *bona fide* β globin promoter and upstream cryptic promoters make use of the same transcription factors, that these factors are in excess of template at lower levels and that, with increasing template, the β globin promoter titrates transcription factors away from cryptic promoters upstream of the cap site. The differences between individuals could then reflect differences in transcription factor levels.

Contrary to a previous report (Bendig & Williams, 1984*a*), *Xenopus* oocytes appear to be a very efficient system for the expression of the *Xenopus* β globin gene in our hands. Comparison of the cap site signal from 1 ng of pXGβ1 with those from known amounts of *Xenopus* β globin mRNA isolated from *Xenopus* erythroblasts (Fig. 2A, lanes 1, 8 and 9) allows us to estimate that β globin transcription in the oocyte occurs at a minimum rate of approximately 50–150 transcripts per gene copy per 24 h. We have found similar transcription rates for the chicken β globin gene in the oocyte. This is an order of magnitude higher than the *Herpes tk* gene (McKnight, Gavis,

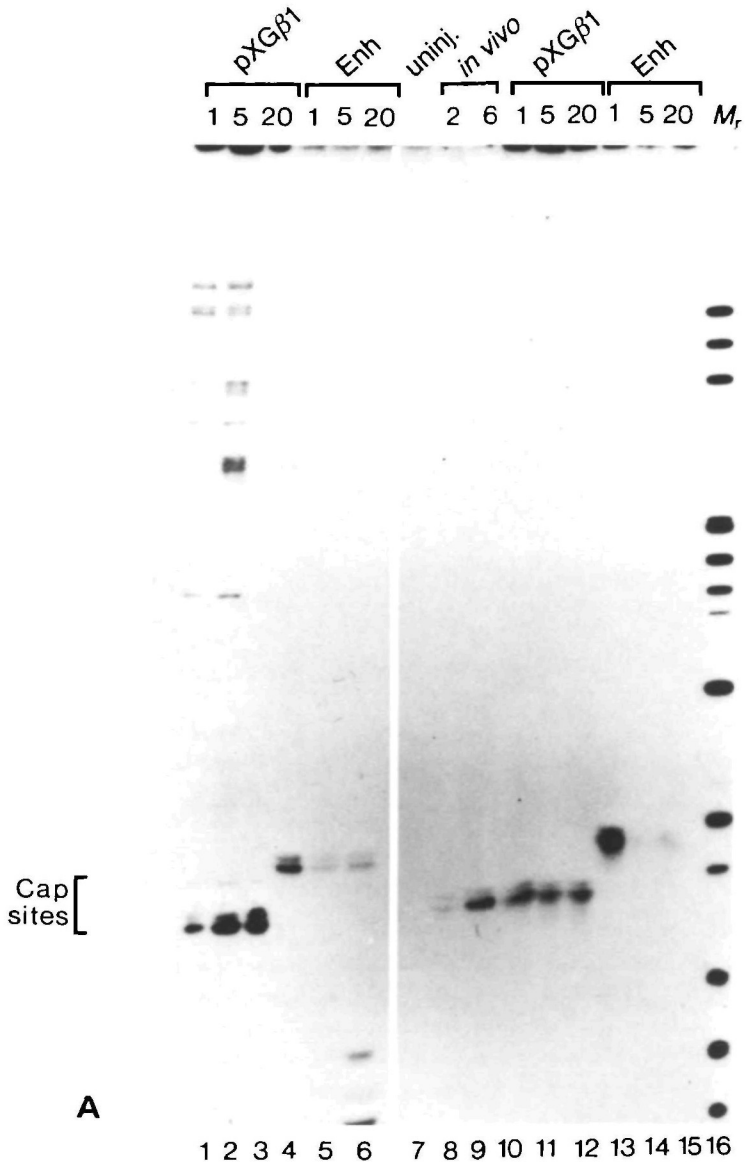

Fig. 2. Primer extension analysis of steady-state RNA synthesized in injected oocytes and controls for injection success. All the data are derived using oocytes from the same individual frog. (A) *Xenopus* β globin transcription. (Lanes 1–3) RNA from two oocytes injected with 1 ng (lane 1), 5 ng (lane 2) and 20 ng (lane 3) pXGβ1. (Lanes 4–6) RNA from two oocytes injected with 1 ng (lane 4), 5 ng (lane 5) and 20 ng (lane 6) pXGβ1RH-enh-B. (Lane 7) RNA from two uninjected oocytes. (Lane 8) 200 ng total cytoplasmic RNA from anaemic *Xenopus* blood equivalent to approx. 2 ng β globin mRNA. (Lane 9) 600 ng total cytoplasmic RNA from anaemic *Xenopus* blood equivalent to approx. 6 ng β globin mRNA. (Lanes 10–12) RNA from five activated oocytes injected with 1 ng (lane 10), 5 ng (lane 11) and 20 ng (lane 12) pXGβ1. (Lanes 13–15) RNA from five activated oocytes injected with 1 ng (lane 13), 5 ng (lane 14) and 20 ng (lane 15) pXGβ1RH-enh-B. (Lane 16) Molecular weight marker, 3′ end-labelled pAT153 digested with *Hpa*II yielding fragment sizes of 634, 494, 407, 244, 240, 219, 205, 192, 162 doublet, 149 (faint), 124, 112, 92, 78, 69 ntds. Note that the longer cap site extension product seen in lanes 4–6 and 13–15 is due to the 12 bp insertion in the first exon of the β1 gene in the construct pXGβ1RH-enh-B. Enh, pXGβ1RH-enh-B; uninj., uninjected oocyte control; M_r, molecular weight marker.

Kingsbury & Axel, 1981), often quoted as the most efficiently transcribed pol II gene in the oocyte, and only two orders of magnitude down on calculated rates of transcription in erythroid cells (Weintraub, 1985).

The SV40 enhancer has little stimulatory effect in Xenopus *oocytes*

In transfected cell lines and in *in vitro* transcription systems the SV40 enhancer stimulates transcription of linked genes and, in most cases, specific initiation at the cap site occurs (Serfling *et al.* 1985). We therefore decided to determine the effect of the SV40 enhancer on *Xenopus* β globin transcription in the oocyte.

In the plasmid pXGβ1RH-enh-B, the β globin gene is linked to a 250 bp enhancer cassette which has been shown to enhance transcription of the *Xenopus* β-globin gene in transfected HeLa cells (Enver *et al.* 1987) and which contains all the sequence motifs recently identified as being necessary for enhancer function in HeLa cells (Zenke *et al.* 1986). When this construct was injected into oocytes over a 1–20 ng range, there was very little upstream initiation at all levels of input DNA (Fig. 2A, lanes 4–6). Although very clear in this experiment, especially for the 1 ng level (lane 4), suppression of upstream initiations was not so apparent when the same titration was performed on oocytes from a different individual (data not shown). Whether this variation reflects factor differences between individuals (see Discussion) or has some other explanation is not yet clear. However, for both individuals, very little stimulation of cap site initiation was observed and in fact at higher levels of template, the signal was reduced. For the experiment shown, allowing for the difference in injection success (Fig. 2B, lanes 4 and 10), little or no enhancement of cap site signal was seen at the 1 ng level but, at higher levels of injected DNA, cap site initiations were suppressed compared to the β globin gene without the enhancer (Fig. 2A, compare lanes 4–6 with lanes 1–3). Two possible explanations for the reduction of transcription at high template levels are enhancer-mediated repression of transcription (Gorman, Rigby & Lane, 1985) or factor competition between the SV40 enhancer cassette and the β globin promoter.

Gorman *et al.* (1985) have presented evidence for enhancer-mediated repression of transcription in undifferentiated embryonal carcinoma cells. However, in their experiments, the repression was relieved by

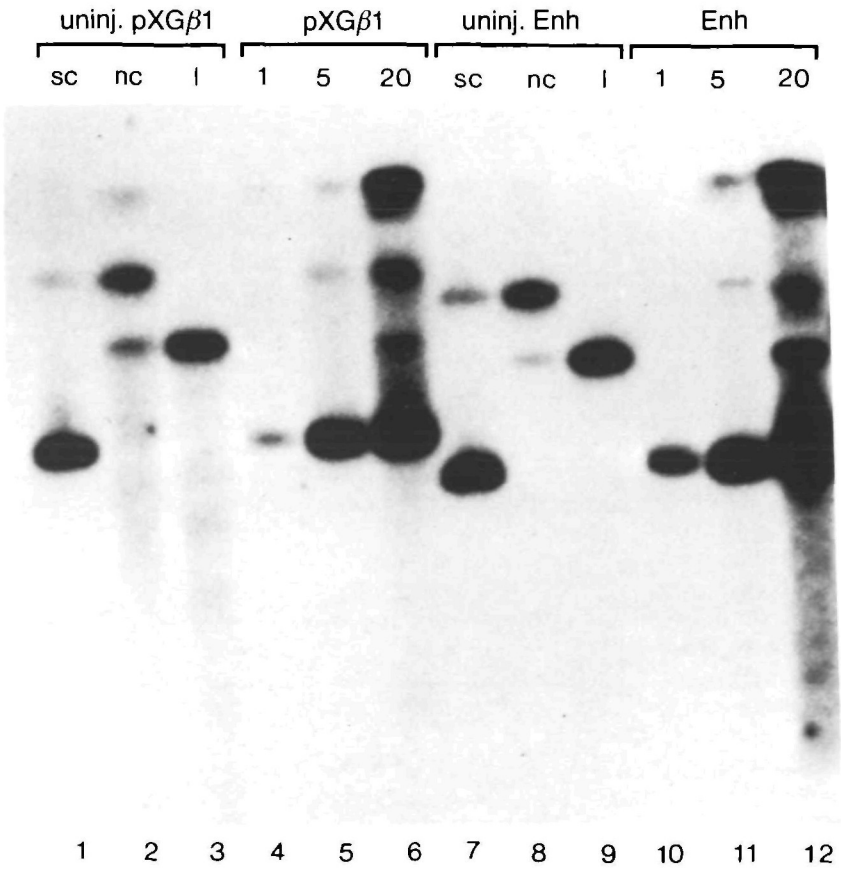

Fig. 2. (B) Southern injection controls. (Lanes 1–3) 5 ng uninjected pXGβ1 markers, supercoiled (sc), nicked circle (nc), linear (l). (Lanes 4–6) DNA recovered from a single oocyte injected with 1 ng (lane 4), 5 ng (lane 5) and 20 ng (lane 6) pXGβ1. (Lanes 7–9) 5 ng supercoiled, nicked circle and linear (respectively) uninjected pXGβ1RH-enh-B markers. (Lanes 10–12) DNA recovered from a single oocyte injected with 1, 5 and 20 ng respectively of pXGβ1RH-enh-B. Uninj., uninjected plasmid control; Enh, pXGβ1RH-enh-B.

increasing template whereas, in the oocyte, repression only becomes apparent at higher template levels. In addition, as we show below, when the enhancer is linked to the SV40 early promoter, no such repression is observed.

An alternative explanation is that the enhancer cassette is competing for a factor required by the β globin promoter. A number of lines of evidence suggests that enhancers and promoters bind common factors (see for example Parslow, Jones, Bond & Yamamoto, 1987) and, in addition to the enhancer, the SV40 fragment used also contains early promoter elements (see Fig. 1) which may bind factors required by the β globin promoter. In order to test this interpretation, we performed a competition experiment (Fig. 3). A constant amount (1 ng) of the β globin-containing plasmid pXGβ1RH (see Fig. 1) was coinjected with increasing amounts of the plasmid pSVENH, which contains the 250 bp enhancer cassette (see Fig. 1) cloned into pUC18. For each injection the amount of DNA injected was held constant at 20 ng by addition of carrier pUC18 DNA. We found that β globin transcription was markedly inhibited by coinjection of pSVENH compared to the control in which the pUC18 vector was coinjected (compare lanes 4 and 7 in Fig. 3, panel A) and that competition was evident even when the enhancer cassette was present at a ratio of one to one (lane 5). This latter situation is equivalent to that in pXGβ1RH-enh-B except that the enhancer cassette and β globin gene are not linked. Thus, the reduction in β globin transcripts seen with increasing levels of pXGβ1RH-enh-B injected (Fig. 2A) probably reflects competition for a common factor which is becoming limiting. The identity of the common factor is currently under investigation in our laboratory.

It has previously been reported that the SV40 enhancer is active in *Xenopus* cells when linked to a heterologous promoter (Weber, de Villiers & Schaffner, 1983) and in particular in *Xenopus* oocytes when linked to its own promoter (Spinelli & Ciliberto, 1985). In view of the small amount of stimulation of β globin transcription we observed, we decided to look carefully at the activity of the SV40 enhancer in the oocyte over a concentration range using CAT constructs in which the CAT gene is driven by the SV40 early promoter. Two constructs were used (Fig. 4A), pSV2cat in which the SV40 enhancer and early promoter are linked to the CAT structural gene and pSV1cat which is similar to pSV2cat but lacks the enhancer (Gorman *et al.* 1982).

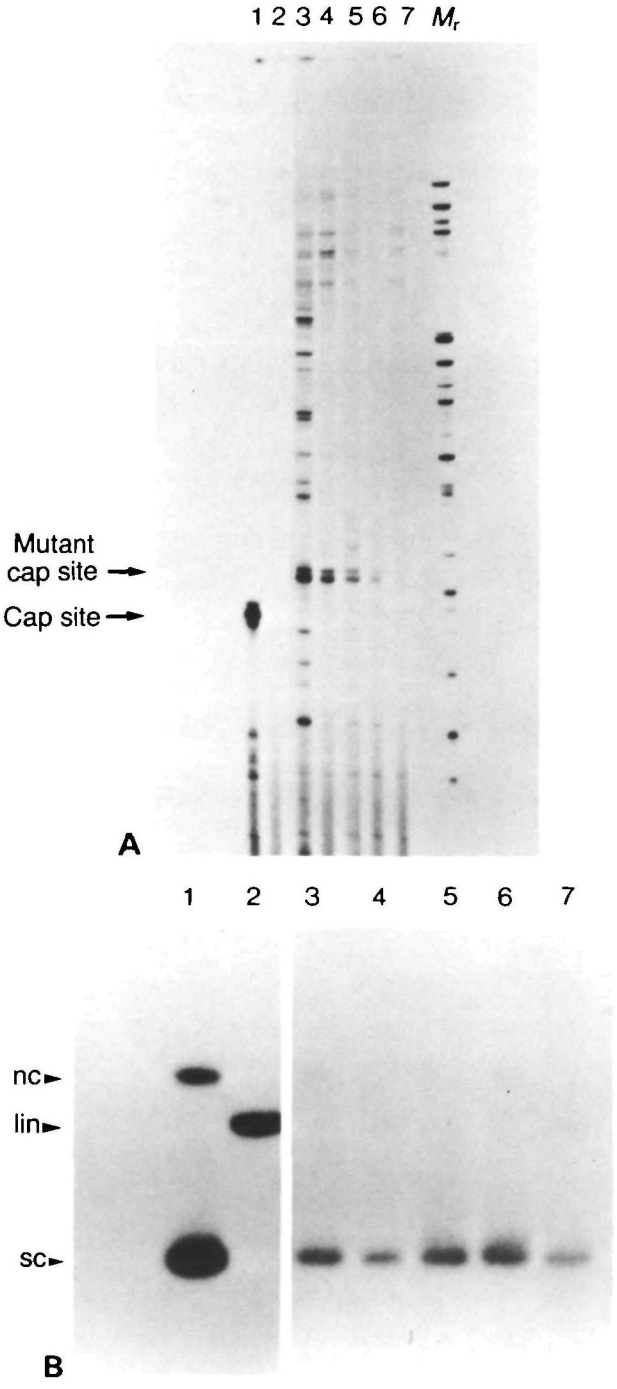

Fig. 3. Competition between the *Xenopus* β globin promoter and the SV40 enhancer-containing cassette. (A) Primer extension analysis. (Lane 1) 175 ng total cytoplasmic RNA from anaemic *Xenopus* blood. (Lane 2) An aliquot of RNA representing a single uninjected oocyte. (Lanes 3–7) RNA representing a single oocyte injected with 1 ng pXGβ1RH: alone (lane 3), +19 ng pUC18 (lane 4), +1 ng pSVENH +18 ng pUC18 (lane 5), +5 ng pSVENH +14 ng pUC18 (lane 6), +19 ng pSVENH (lane 7). M_r is a molecular weight marker, 3′ end-labelled pAT153 digested with *Hpa*II. (B) Southern transfer analysis of injected DNA. (Lanes 1, 2) 5 ng uninjected pXGβ1RH markers; (lane 1) supercoiled (sc) and nicked circular (nc); (lane 2) linear (lin). (Lanes 3–7) Aliquots representing DNA recovered from a single oocyte injected with the components detailed in lanes 3–7, panel A. For clarity, a β globin-specific probe was used (see Materials and methods).

We found that the enhancer stimulated CAT expression at all levels of injected template (Fig. 4A, compare lanes 2, 4 and 6 with 3, 5 and 7). However, quantification of the CAT assay by scintillation counting revealed that the enhancement was only of the order of 2- to 5-fold over the whole range. Using similar constructs, Spinelli & Ciliberto (1985) have claimed an approx. 100-fold enhancement relative to an extremely low level of CAT expression without the enhancer. Since no injection controls were presented, it is very difficult to evaluate the difference between their results and ours. However, in agreement with our data, other studies suggest that deletion of the enhancer has little effect on early gene transcription in *Xenopus* oocytes (Contreras *et al.* 1982). This low level of enhancement could reflect the reported host range effect of the SV40 enhancer (de Villiers & Schaffner, 1983) or may be a consequence of the high level of enhancer-independent transcription, a phenomenon also noted in the experiments with undifferentiated embryonal carcinoma cells (Gorman *et al.* 1985).

A surprising result was the decrease in CAT expression with increasing template for the pSV constructs (Fig. 4A, lanes 2–7). This effect is not mediated by the enhancer since pSV1cat contains no enhancer. One possible explanation is that transcription factors for the SV40 early promoter are limiting in the oocyte and that their binding is non-cooperative (see Discussion). Thus, at higher levels of injected DNA, these factors would be non-productively distributed over an excess of template molecules resulting in few templates with the correct complement of factors necessary for full transcriptional activity.

Aberrant upstream initiations are eliminated in activated oocytes

When the *Xenopus* β globin gene was injected into *Xenopus* unfertilized eggs which, in contrast to oocytes, are competent for DNA replication, transcription occurred only from the cap site, albeit at a very low level (Bendig & Williams, 1984*a*). Based on the use of aphidicolin to block DNA synthesis, the authors suggested that the fidelity of transcription was due to replication. The possibility that DNA replication might affect β globin expression was further suggested by the observation that, in transfected HeLa cells, enhancer-dependent *Xenopus* β globin expression is stimulated by replication (Enver *et al.* 1987). In fact, when transfection is effected by dextran, transcriptional activation is absolutely dependent on both a linked enhancer and the passage of a replication fork through the gene. We decided, therefore, to investigate the effect of replication on *Xenopus* β globin transcription in oocytes in which DNA synthesis had been induced.

Xenopus oocytes do not have a functional replication system but, when incubated with progesterone and subsequently activated by a needle prick, replication ensues (Harland & Laskey, 1980). pXGβ1 and pXGβ1RH-enh-B were injected into oocytes over a 1–20 ng range, incubated overnight with progesterone, prick activated and then collected for analysis 6 h later.

First, we established that DNA replication had occurred as a result of the hormone treatment and prick activation. Injected plasmids recovered from activated oocytes were digested with *Mbo*I, a restriction enzyme which digests doubly replicated, demethylated DNA only, and compared with the same undigested samples (Fig. 5). For each injection, replication was confirmed by a reduction in supercoiled monomer accompanied by the appearance of a characteristic pattern of *Mbo*I digestion products (Fig. 5, lanes 4–9 and 14–19) when compared to a *Sau*3AI digest of injected DNA (lanes 10 and 20). Extra bands in lanes 10 and 20 are due to partial digestion by *Sau*3AI. In a control experiment, we found that when DNA recovered from nonactivated injected oocytes was digested with *Mbo*I the typical pattern of digestion products was absent whereas, *Dpn*I, which digests only nonreplicated DNA converted all of the DNA to these characteristic digestion products (data not shown).

Primer extension analysis of the RNA in these same samples is shown in Fig. 2A lanes 10–15. In this experiment, RNA from five oocytes was assayed compared to only two oocyte equivalents in the non-prick-activated series. Oocytes from the same individual were used for both experiments. Taking the difference in oocyte equivalents into account, comparison of the cap site signals from activated and nonactivated oocytes revealed that no significant stimulation of cap site initiation was achieved as a result of the induction of DNA replication either in the presence or absence of the enhancer (Fig. 2A, compare lanes 1–6 with lanes 10–15). Thus the transcriptional stimulation by replication in the HeLa system is not mirrored in the oocyte.

However, at all levels of injected pXGβ1, upstream initiations were suppressed and transcription occurred almost exclusively from the cap site in contrast to the results obtained in nonactivated oocytes (compare Fig. 2A lanes 1–3 and 10–12). This is not simply due to replication bringing template numbers above the critical 5 ng level (when upstream starts are repressed in nonactivated oocytes). Comparison of the amounts of DNA recovered from prick-activated oocytes with the uninjected control, loaded at 5 ng, revealed that in two out of three injections 5 ng or less were recovered (compare supercoiled monomer in lanes 4 and 6 with lane 1,

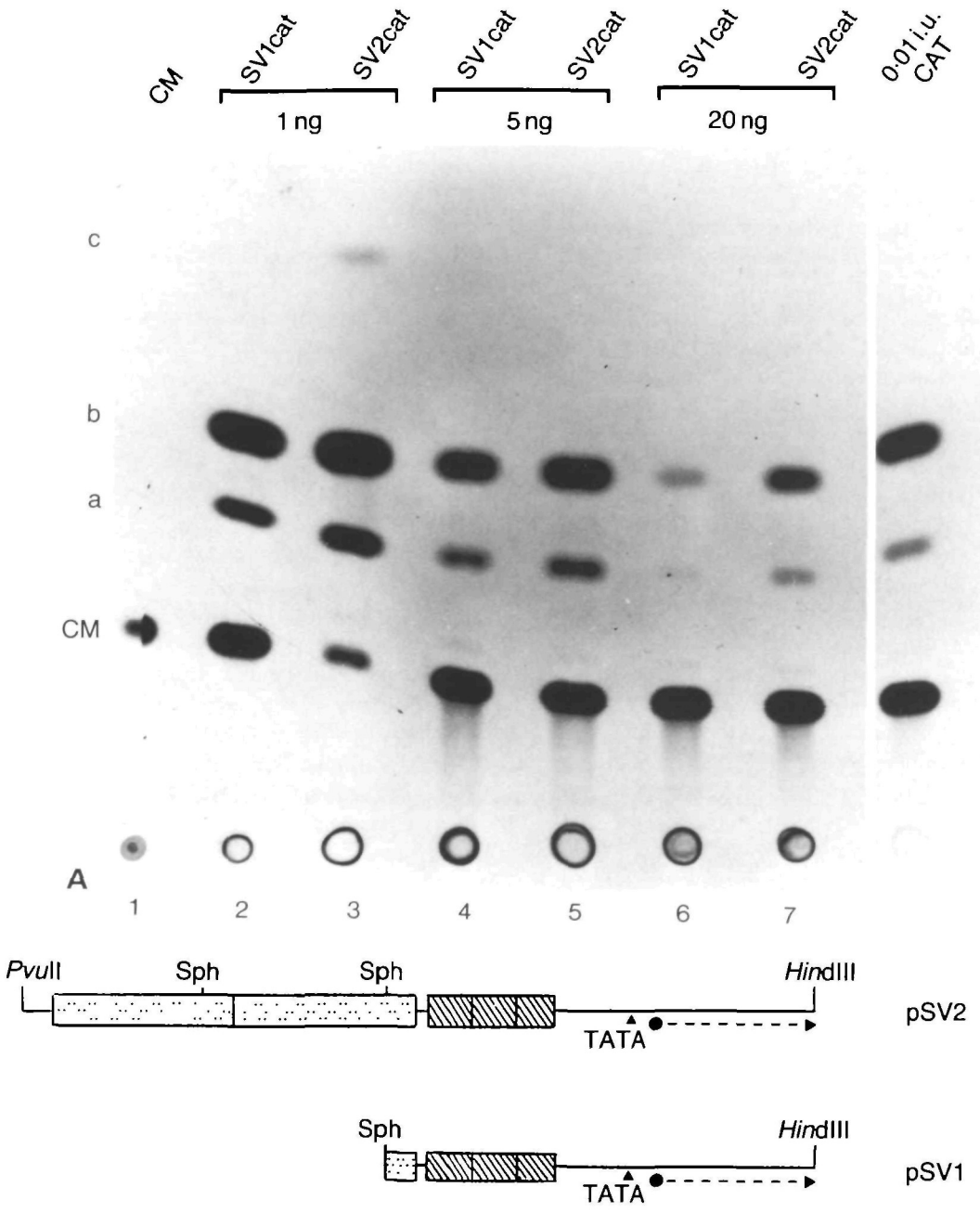

Fig. 4. CAT assays and Southern injection controls. (A) CAT assays. 20 μl samples of extracts prepared from injected oocytes were assayed for 2 h at 37 °C as described in Materials and methods. (Lane 1) CM, ^{14}C-chloramphenicol control; (lanes 2, 3) extracts from two oocytes injected with 1 ng pSV1cat or pSV2cat, respectively (lanes 4, 5) extracts from two oocytes injected with 5 ng pSV1cat or pSV2cat, respectively; (lanes 6, 7) extracts from two oocytes injected with 20 ng pSV1cat or pSV2cat, respectively; (lane 8) control CAT 0·01 i.u. (P.L. Biochemicals, Inc.). a,b, mono-acetylated chloramphenicol forms; c, di-acetylated chloramphenicol. Diagrams of pSV2cat and pSV1cat are shown below (Gorman *et al.* 1985).

Fig. 5). Yet, at these levels of injected DNA in nonactivated oocytes, upstream initiations are clearly present. This result confirms the effects seen in *Xenopus* unfertilized eggs (Bendig & Williams, 1984*a*).

Furthermore, in activated oocytes, β globin transcription from the cap site was independent of template levels unlike the situation in nonactivated oocytes (compare lanes 1–3 and 10–12 in Fig. 2A). This is true for both replicated and unreplicated templates since the levels of both increase over the titration range (Fig. 5). The increase in fidelity of transcription and lack of dependence on template numbers is consistent with saturation of the available transcription factor pool with β globin promoters in a

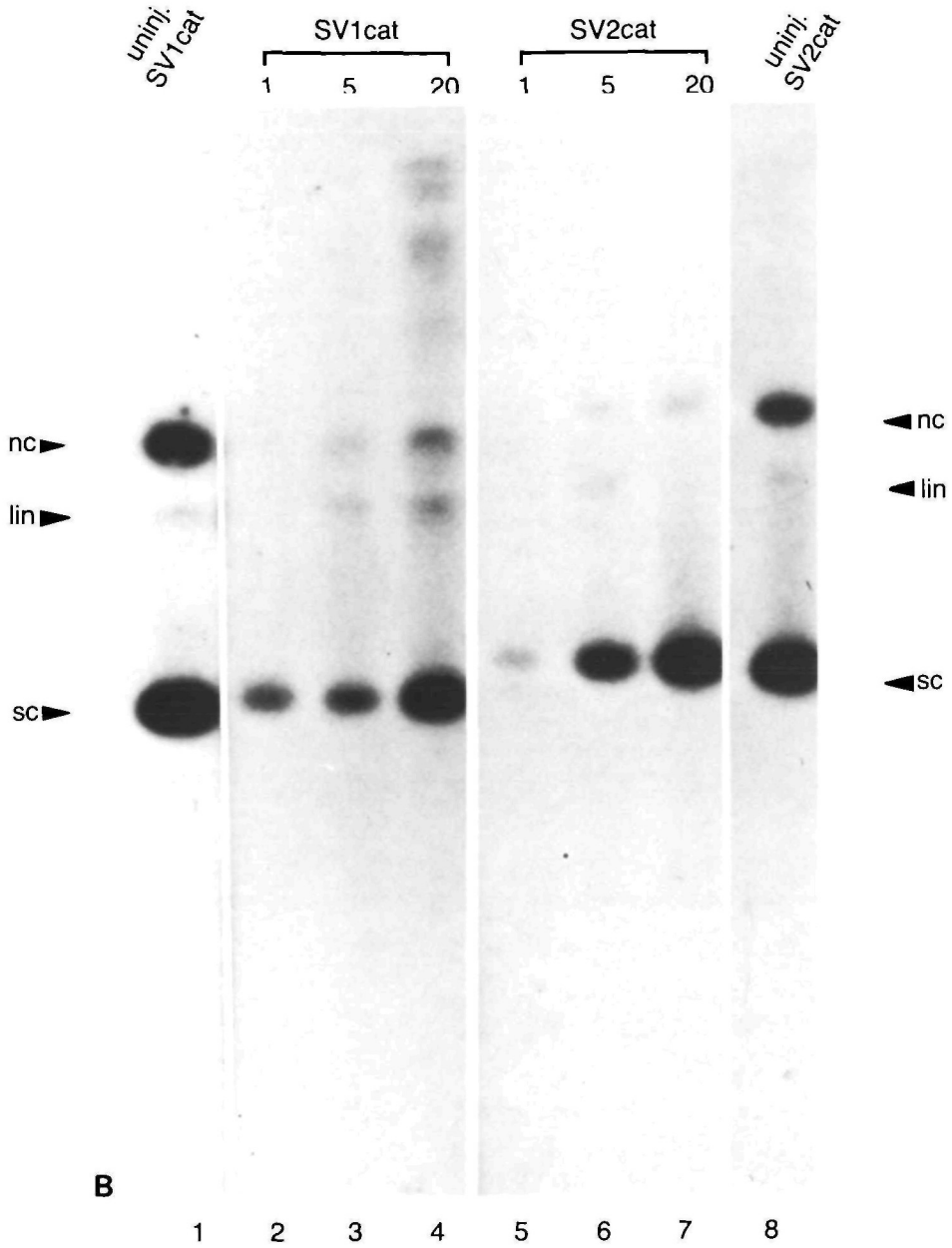

Fig. 4. (B) Southern injection controls. (Lane 1) Uninjected pSV1cat marker, the positions of supercoiled (sc), nicked circle (nc) and linear (lin) forms are indicated. (Lanes 2–4) DNA recovered from the equivalent of a single oocyte injected with 1, 5 and 20 ng, respectively, of pSV1cat. (Lanes 5–7) DNA recovered from the equivalent of a single oocyte injected with 1, 5 and 20 ng, respectively, of pSV2cat. (Lane 8) Uninjected pSV2cat marker.

manner similar to that seen at the top end of the titration in nonactivated oocytes. The fact that the level of transcription does not decrease with increasing template, as seen with the SV40 early promoter, would then suggest that, for the β globin promoter, factor binding is cooperative. Thus, these data may be reflecting a reduction in the availability of transcription factors on maturation and activation of the oocyte. The greater suppression of cap site signal at high template levels for pXGβ1RH-enh-B (Fig. 2A, lanes 14 and 15) is consistent with this hypothesis, if, as for the nonactivated oocyte, we assume there to be competition for a common factor.

Discussion

It has recently been demonstrated that β globin genes have an erythroid cell-specific enhancer located 3′ of

the gene (Hesse *et al.* 1986; Choi *et al.* 1986; Emerson *et al.* 1987; Kollias *et al.* 1987). A DNase I hypersensitive site has been found in an equivalent position in the *Xenopus* β globin gene in *Xenopus* erythrocyte nuclei (Patient, Harris, Walmsley & Williams, 1983; Brewer *et al.* unpublished data), suggesting the presence of a similarly located enhancer in *Xenopus*. The enhancer function can be substituted in non-erythroid cells by the more promiscuous viral enhancers (Serfling *et al.* 1985). We show here, however, that the β globin gene is highly active in the absence of the erythroid-specific factors which might be presumed to activate the β globin enhancer, and also without the enhancer function being supplied by viral sequences. Although enhancer-independent β globin transcription has been demonstrated *in vitro*, the efficiency is

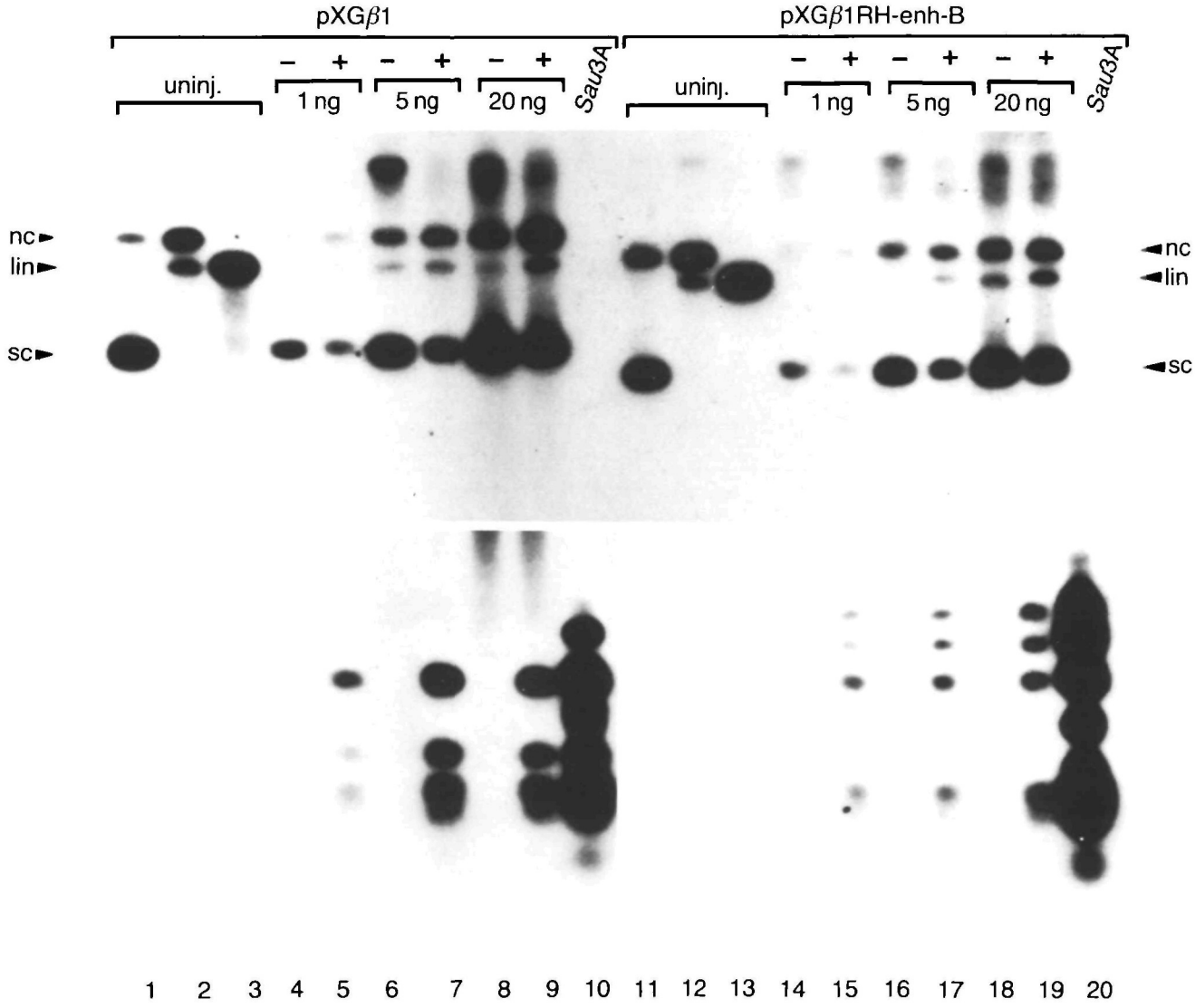

Fig. 5. Southern injection controls and assay for DNA replication in activated oocytes. Samples of DNA extracted from injected activated oocytes were divided into two aliquots. One was digested with *Mbo*I (+) the other was not (−). Samples were electrophoresed on an agarose gel alongside a *Sau*3AI digest of DNA extracted from nonactivated oocytes injected with the relevant plasmid and together with uninjected plasmid controls. Samples were blotted onto nitrocellulose and probed with nick-translated vector. The *Mbo*I digested samples (+) measure replication whilst the undigested samples (−) represent injection success. (Lanes 1–3) 5 ng uninjected supercoiled (sc), nicked circle (nc) and linear (lin) pXGβ1 respectively. (Lanes 4–9) Aliquots representing DNA recovered from a single oocyte injected with 1, 5 and 20 ng respectively of pXGβ1 and either undigested (−) or digested (+) with *Mbo*I. (Lane 10) DNA recovered from a single nonactivated oocyte injected with 5 ng pXGβ1 and digested with *Sau*3AI (note this digest is partial). (Lanes 11–20) As for lanes 1–10 except pXGβ1RH-enh-B was injected. The lower half of the figure is a longer exposure of the same filter which was required to pick up the weaker signals from the *Mbo*I/*Sau*3AI digestion products.

orders of magnitude below that reported here (Luse & Roeder, 1980). One possible explanation may be that the oocyte has the factors necessary to activate the β globin enhancer. However, a β globin gene construct lacking the 3′ hypersensitive site sequences displays no loss of transcriptional activity in the oocyte (see Fig. 3A for example). We conclude that the activation step provided by the enhancer is somehow bypassed in the oocyte. If enhancers function to increase local transcription factor concentration (Yamamoto, 1985), then their need may be obviated in the oocyte due to transcription factor abundance. A greater understanding of this enhancer-independent transcription may provide clues to the steps in gene activation that require enhancer involvement in other cells.

Variability in the fidelity of transcription of tissue-specific RNA polymerase II-dependent genes between individual frogs has been reported (Jones, Richter, Weeks & Smith, 1983; Bendig & Williams, 1984*a*; Partington *et al.* 1984). By performing experiments on a single individual, we have shown that, within one individual, transcriptional fidelity varies with the amount of injected template. It is therefore possible that the observed variability between individuals is due to differences in transcription factor levels. Consistent with this interpretation, we have found that whilst maximal cap site and minimal upstream initiations occur at 20 ng of injected template for one individual (Fig. 2), this situation is not achieved until 80 ng for two other individuals, whilst in yet two more individuals upstream initiations were eliminated at the 1 ng level.

The results with the SV40–CAT constructs may also be reflecting transcription factor availability in the oocyte. The reduction in CAT synthesis seen with increasing levels of injected pSV1cat suggests that (1) factors interacting with the SV40 early promoter are limiting in the oocyte, (2) their binding is non-cooperative and (3) the binding of more than one factor molecule is required for functional transcription. The enhancerless SV40 early promoter contains two classes of sequence element: the GC boxes found in the 21 bp repeat and the AT-rich segment. Since deletion of the AT element does not dampen early transcription (McKnight & Tjian, 1986), the GC box-binding factor, Sp1 (Dynan & Tjian, 1983; McKnight & Tjian, 1986), is implicated as the limiting factor in the oocyte. As suggested for the limiting oocyte factor, Sp1 does indeed bind non-cooperatively to its target sequences and furthermore, for maximal early promoter activity, Sp1 must be bound to at least three of the six GC boxes found in the early promoter (Gidoni *et al.* 1985). Sp1 is also required for expression of the *Herpes* virus *tk* gene (Gidoni *et al.* 1985), and the promoter elements of this gene were identified using the *Xenopus* oocyte as the transcriptional assay system (McKnight & Kingsbury, 1982). A titration of the *Herpes tk* gene in the oocyte has not, to our knowledge, been reported but we would predict that, since Sp1 appears to be limiting in the oocyte and the binding of two protomers is required for full promoter function (McKnight, 1982), *tk* expression would be reduced as template numbers increased.

The contrast between the template level requirements for the promoters of the SV40 early gene (transcription decreases with increased template) and the *Xenopus* β globin gene (transcription increases with increased template) cautions that, for optimal transcription of any gene, a titration of template must be carried out in order to reconcile the transcription factor requirements of the gene promoter to factor levels within the oocyte. Specificity of transcription can also be improved by maturation and activation of the oocyte. These considerations must be borne in mind when, for example, using the oocyte as a complementation assay to identify gene regulatory proteins in nuclear extracts. Experiments of this type are in progress to see if erythroid nuclear proteins can provide the extra two orders of magnitude necessary to mimic *in vivo* expression.

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Note added in proof

Since submission of this manuscript, a titration of the Herpes *tk* gene in the oocyte has appeared in the literature (Pena, P. & Zasloff, M. (1987). *Cell* **50**, 613–619), showing that *tk* transcription is indeed reduced as template numbers increase.