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Genetics

Transformation of frog embryos with a rabbit β-globin gene

(gene transfer/microneedle injection/DNA replication/gene regulation)

SANDRO RUSCONI AND WALTER SCHAFFNER

Institut für Molekularbiologie II der Universität Zürich, Hönggerberg, CH-8093 Zürich, Switzerland

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ABSTRACT **In order to study the fate and possible expression of foreign DNA during embryogenesis of the frog *Xenopus laevis*, we have injected a rabbit β-globin gene into fertilized *Xenopus* eggs. Frog embryo DNA was extracted at various stages of development, fractionated by agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized to labeled β-globin recombinant plasmid DNA. It was found that the injected DNA replicated extrachromosomally, reaching, at gastrula stage, a level equivalent to a 10- to 200-fold amplification of input DNA. At later stages, a majority of the foreign DNA was degraded, but a small fraction was maintained. Six-week-old tadpoles as well as six-month-old frogs contained an average of 3–10 copies of the rabbit globin gene per cell. Most of these persisting globin genes were present as long tandem repeats and comigrated in agarose gel electrophoresis with high molecular weight *Xenopus* DNA. Analysis of globin gene expression by S1 nuclease mapping showed that the rabbit β-globin promoter was recognized in the frog embryo and that the transcripts were correctly spliced.**

Oocytes and eggs of the frog *Xenopus laevis* have been used for many years as experimental systems for studying the translation of injected mRNAs (reviewed in ref. 1). More recently, microneedle injection of DNA into *Xenopus* oocytes has provided a functional test to analyze the biological activity of cloned genes (2–5), a concept named "surrogate genetics" (6). Unfertilized eggs and egg extracts are reported to promote replication of injected DNA (7–12). Fertilized eggs injected with DNAs (13, 14) or rabbit globin mRNAs (15) are able to continue developing into apparently normal tadpoles.

In the present studies, we have injected a genomic rabbit β-globin gene (16) into fertilized eggs of *Xenopus laevis* and have studied its fate throughout development. We have chosen the rabbit gene for the following reasons: (*i*) rabbit globin DNA can be identified by hybridization in the presence of a large excess of *Xenopus* DNA; (*ii*) intracellular rabbit β-globin protein is not toxic for *Xenopus* embryogenesis (15); and (*iii*) the molecular structure of the cDNA plasmid (ref. 17; recloned by F. Meyer, Zürich) and of the genomic gene (16, 18, 19) were known in detail. Our results indicate that the injected genes replicate and are correctly transcribed during early embryogenesis, and that they persist in young frogs.

MATERIALS AND METHODS

Injected DNAs. The genomic rabbit β-globin gene clone λCh4A-Rβ1 was obtained from T. Maniatis, Pasadena, CA [refs. 16 and 18; referred to as the "genomic" clone, as distinguished from the "gnomic" Zürich clone independently isolated by C. Weissmann and his colleagues (20)]. The globin gene was excised from λCh4A-Rβ1 and recloned as follows (cf. Fig. 1): (*i*) pP+, the 6.5-kilobase pair (kb) *Pst* I globin gene fragment, was inserted into the *Pst* I site of the plasmid pBR322 (21, 22) in opposite orientation with respect to the β-lactamase gene; (*ii*) pSVK+, the 4.7-kb *Kpn* I globin gene fragment, was inserted into the single *Kpn* I site found in the simian virus 40 (SV40) "late" region of the previously constructed recombinant plasmid pBSV-early (23); (*iii*) pSVKd+, the same as pSVK+ but with two globin gene inserts in tandem; (*iv*) pSVKd−, as pSVKd+ but with two globin genes in the same orientation as the β-lactamase gene; (*v*) pJKd−, the 4.7-kb *Kpn* I globin gene fragment cloned as a dimer insert into the plasmid pJC-1 (pJC-1 is a derivative of pBR322 containing a *Kpn* I site instead of the *Eco*RI site, a gift of J. Jenkins, London); (*vi*) monomeric circles of the globin gene *Kpn* I fragment free of plasmid DNA were prepared as described for histone genes (24). Dimeric circles were made from the plasmid pJKd−*, a derivative of pJKd− in which the *Kpn* I site between the two globin genes had been destroyed by *Kpn* I cleavage, S1 nuclease digestion, and religation. Recombinant plasmids between pBR322 and globin gene DNA were constructed by standard cloning procedures (25, 26). All work involving recombinant plasmids was done under conditions conforming to the standards outlined in the National Institutes of Health guidelines for recombinant DNA research.

Microinjection. Artificially fertilized eggs of *X. laevis* were prepared as described by Billett and Wild (27). Fertilized eggs were decapsulated for 2 min in a solution of 3% (wt/vol) L-cysteine, 0.1% papain, and 0.23 M NaOH and then washed five times with 2 ml of fresh Barth's solution. Healthy fertilized eggs were injected within 1 hr, before the first cleavage event occurred. The microinjection technique was essentially the same as used for oocytes (2), except that the centrifugation step was omitted. Each egg was injected with a glass capillary needle from which there was a constant flow of DNA. The needle was inserted through the vitellin membrane at a 45° angle; unless otherwise stated, 250 pg of DNA (about 2×10^7 genes) in a volume of 10 nl was deposited within about 4 sec at the center of the egg. Two hours after injection, embryos were transferred from Barth's medium to sterile "aged" water (27) plus antibiotics (GIBCO) and incubated at 18°C.

Analysis of DNA and RNA. Samples of 5–10 embryos were collected immediately after injection and at the indicated stages of development according to Daudin (28). Embryos or tadpoles were homogenized at 0°C in 10 mM EDTA, 100 mM Tris·HCl (pH 7.5), 300 mM NaCl, proteinase K at 1 mg/ml, and 2% (wt/vol) sodium dodecyl sulfate. Frogs were treated the same way except that the gut was removed prior to homogenization. After a 30-min incubation at room temperature, the homogenate was extracted three times with phenol and two times with chloroform, and the nucleic acids were precipitated with 2 vol of ethanol. For Southern blotting (29, 30), DNA isolated from embryos at progressive stages of development was electrophoresed

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Abbreviations: kb, kilobase pairs; SV40, simian virus 40.

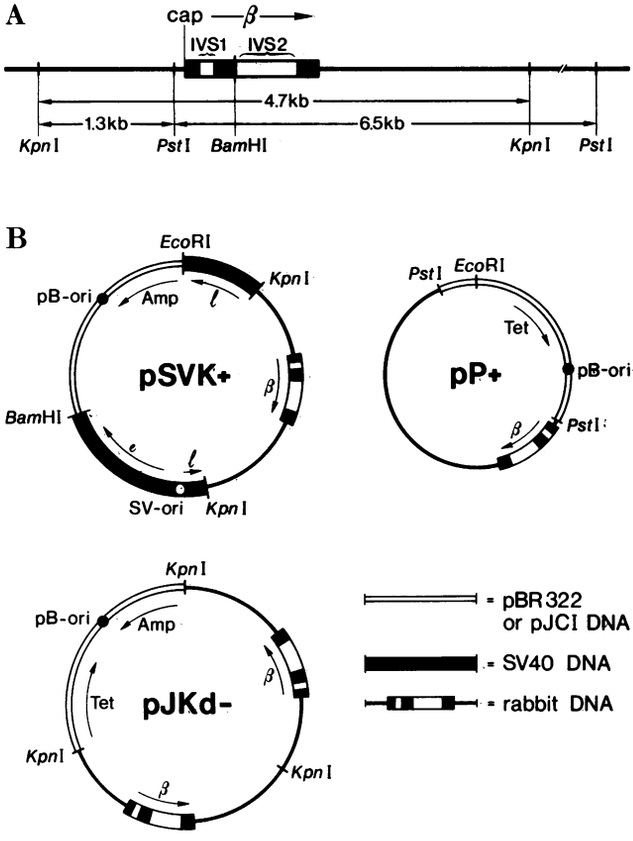

FIG. 1. (*A*) Schematic representation of the genomic rabbit hemoglobin β-1 gene (16, 18). The black line is noncoding DNA, the black bars are coding regions, and the open bars are intervening sequences (IVS1 and IVS2). (*B*) Recombinant DNAs containing the β-1 globin gene. Symbols not identified in the figure are: Tet, tetracycline resistance genes; Amp, β-lactamase gene; pB-ori and SV-ori, plasmid and SV40 origins of replication; *e*, SV40 early region; *ℓ*, late region.

through a 0.8% agarose gel and transferred to nitrocellulose as described in ref. 30. The transferred DNA was hybridized to a ^{32}P-labeled nick-translated probe of pBR322 recombinant plasmid containing the rabbit β-globin gene. S1 nuclease mapping of transcripts was done as described by Weaver and Weissmann (ref. 31; Fig. 4*A*). Splicing of the first intron was detected by using a *Pst* I/*Bam*HI restriction fragment (position −99 to +480; ref. 19) obtained from the genomic β-globin gene and ^{32}P-labeled at the *Bam*HI site as the hybridization probe. The probe DNA was denatured, hybridized to unlabeled RNA, and treated with S1 nuclease, and the products were analyzed by gel electrophoresis. Genuine rabbit β-globin mRNA (a gift of S. Nagata and C. Weissmann, Zürich) protected a labeled DNA fragment of 208 nucleotides spanning the region from the acceptor site of the first intron to the *Bam*HI site (position 272 to 480). In order to map the 5′ end of the transcripts, a globin gene clone from which the first intron had been deleted (designated Z-pBR/RcβG-A4, obtained from H. Weber, Zürich) was labeled at the *Bam*HI site and used as a hybridization probe. Unlabeled β-globin mRNA protected a DNA fragment of 354 nucleotides, from the cap site to the *Bam*HI site (position 1 to 480 of the genomic gene).

RESULTS

Toxicity of the Injected DNA. pBR322–β-globin recombinant DNA was injected into a total of 5239 fertilized eggs, either in a supercoiled form or after cutting at the single *Ava* I site within pBR322. In a large fraction of the embryos, development was not affected when up to 250 pg of DNA (about 2×10^7 genes) was injected per egg. Larger DNA quantities were increasingly toxic. With supercoiled DNA, 40%, 19%, 5%, and less than 1% of the embryos reached the swimming larva stage after injection of 0.25 ng, 0.5 ng, 1 ng, or 2 ng of supercoiled DNA, respectively. Linear DNA was found to be less toxic than supercoiled DNA (59%, 30%, 16%, and 7% swimming larvae after injection of 0.25 ng, 0.5 ng, 1 ng, or 2 ng of DNA, respectively).

Replication and Persistence of Injected DNA. Total embryonic DNA was extracted at various stages during development, fractionated by agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized to labeled rabbit β-globin recombinant plasmid DNA. It was found that supercoiled DNA was converted into a relaxed but covalently closed circular form immediately after injection. A few hours later, supercoiled DNA reappeared, as was found with circular DNA injected into frog oocytes (32). In addition, there was a large net increase of injected DNA sequences, reaching a maximal copy number at late gastrula (Fig. 2). The extent of DNA replication varied from one experiment to another, ranging from a 10- to 200-fold amplification of the input DNA, as determined by scintillation counting of membrane filter strips from Southern blots and dot hybridizations (e.g., egg 7 cpm, blastula 466 cpm, late gastrula 1480 cpm, feeding larva 76 cpm). We found that efficient replication depended on the amount of DNA injected. Whereas there was little replication with 125 pg of foreign DNA per egg (data not shown), extensive replication was observed with 250 pg or, in particular, 500 pg per egg (Fig. 2). Replication was not significantly different when the injected DNA was supercoiled or linear, or when it contained pBR322, an SV40 replicon, or both (Fig. 2; unpublished observations). As can be seen in Fig. 2*A*, globin gene sequences appeared in high molecular weight bands, but there was also a large increase of DNA migrating in

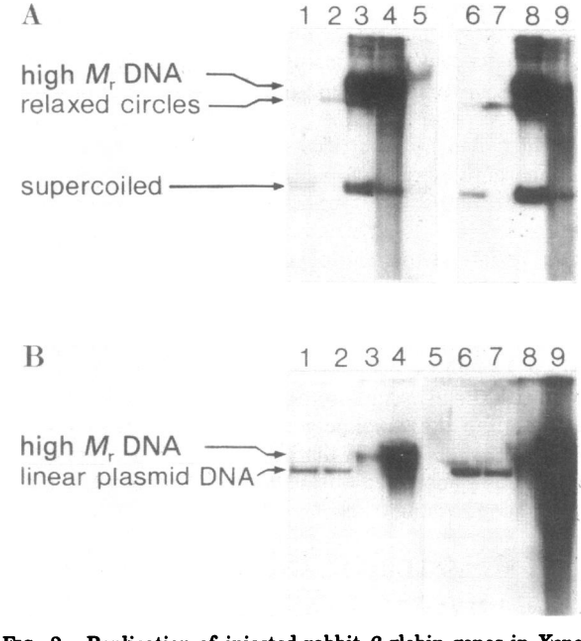

FIG. 2. Replication of injected rabbit β-globin genes in *Xenopus* embryos. DNA was extracted from injected embryos at various developmental stages, and the DNA equivalent of one embryo was electrophoresed through agarose gel, transferred to nitrocellulose, hybridized to ^{32}P-labeled globin gene probe, and autoradiographed. (*A*) Injection of supercoiled recombinant DNA (pP+). (*B*) Injection of linear recombinant DNA (pSVK+). Slots 1–5, 250 pg of DNA injected per egg; slots 6–9, 500 pg of DNA injected per egg. Slots 1 and 6, sample of DNA equivalent to amount injected; slots 2 and 7, DNA extracted from eggs 2 min after injection; slots 3 and 8, DNA from late blastula (stage 9); slots 4 and 9, DNA from late gastrula (stage 13); slot 5, DNA from swimming larvae (stages 41–45). M_r, molecular weight.

the position of supercoiled input plasmid DNA. The latter indicates that the injected DNA at this stage replicated autonomously rather than as part of *Xenopus* chromosomal DNA. After the gastrula stage, most of the foreign DNA was degraded. However, the remaining DNA was found to persist for many months. In gel electrophoresis, globin gene–pBR322 recombinant DNA migrated together with high molecular weight *Xenopus* DNA. Six-week-old tadpoles as well as 6-month-old frogs contained an average of 3–10 rabbit β-globin gene copies per cell, as judged from the intensity of the autoradiographic signals in Southern blots (Fig. 3). This calculation was based on a DNA content of 6 pg per diploid *Xenopus* cell (14) and a size of 13 kb for the pJKd− plasmid containing two globin genes.

DNA from tadpoles and frogs originally injected with supercoiled pJKd− plasmid was analyzed by restriction endonuclease digestion and Southern blotting (29, 30). We concluded that most of the persisting foreign DNA was present as head-to-tail tandem repeats of the original circular input DNA, because the persisting DNA was present in a high molecular weight form and yet gave the same restriction pattern as the input DNA: Digestion of the DNA with *Bst*EII, which does not cut the input pJKd− DNA, gave a hybridizing band of very high molecular weight (Fig. 3). The possibility that this high molecular weight band was the result of trapping of input plasmid DNA within high molecular weight *Xenopus* DNA was eliminated by the following reconstruction experiment: pJKd− plasmid DNA was mixed with *Xenopus* DNA, digested with *Bst*EII, and electrophoresed in parallel with tadpole DNA from injection experiments. The plasmid DNA migrated as supercoiled and relaxed circles and not with the high molecular weight DNA (Fig. 3). Digestion of the DNA with *Hin*dIII (Fig. 3), *Ava* I, or *Sal* I (data not shown), all cutting the input plasmid once but at different positions, yielded a prominent band at the position of linear pJKd− DNA. Digestion with *Eco*RI and *Pst* I gave the same fragment pattern for both the persisting DNA and the input plasmid DNA (data not shown). So far, the findings are also compatible with the assumption that the foreign DNA persisted as catenanes (interlocked circles; ref. 33) of the circular input plasmid DNA. This seems unlikely because of digestion experiments with dilute DNase I: In a reconstruction experiment circular pJKd− DNA was mixed with *Xenopus* DNA and treated with increasing concentrations of DNase I. Southern blotting showed that the plasmid circles were nicked and then opened to linear form III DNA before they were degraded to smaller fragments. Under the same conditions, tandem multimers would be expected to yield a smear of random degradation products only. In fact, Southern blot analysis of DNA from tadpoles revealed that the persisting β-globin–pBR322 sequences were converted by DNase I from high molecular weight DNA to heterogenous smaller fragments with no detectable intermediate of linear plasmid DNA (data not shown).

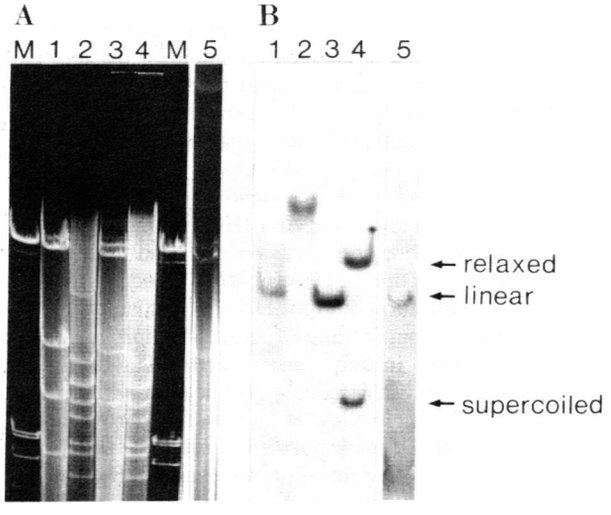

FIG. 3. Analysis of persisting rabbit β-globin genes in tadpoles and frogs. DNA was extracted from tadpoles and frogs, digested with restriction endonuclease, and analyzed as described in *Materials and Methods*, except that a 0.45% agarose gel was used in order to get high resolution of large molecular weight DNA fragments. One microgram of bacteriophage λ DNA was added to each DNA sample to test for complete digestion. (*A*) Gel stained with ethidium bromide. (*B*) Autoradiograph of the same gel after Southern blotting and hybridization to globin gene probe. Slots 1, 2, and 5, DNA from injection experiments with supercoiled pJKd− recombinant plasmid; slots 1 and 2, 4-μg DNA samples from a pool of ten 6-week-old tadpoles; slot 5, 4-μg DNA sample from a pool of three 6-month-old frogs; slots 3 and 4, reconstruction experiment in which 100 pg of pJKd− DNA was mixed with *Xenopus* DNA prior to digestion; slots 1, 3, and 5, restriction digestion with *Hin*dIII (cuts pJKd− DNA once); slots 2 and 4, digestion with *Bst*EII (does not cut pJKd−); slot M, bacteriophage λ marker DNA cut with *Eco*RI and *Hin*dIII.

Correct and "Incorrect" Transcription of the Rabbit β-Globin Gene. Transcription of the injected rabbit β-globin gene was detected by the S1 nuclease mapping technique (31). DNA from the genomic clone was ^{32}P-labeled in the minus strand at the *Bam*HI site and hybridized to RNA extracted from the transformed *Xenopus* gastrulae (Fig. 4*A*). After S1 nuclease digestion, denaturation, and gel electrophoresis, a strong band, approximately 208 nucleotides in size, was found with RNA from late gastrulae (Fig. 4*B*, lanes 1 and 2). The same band appeared in reconstruction experiments with authentic rabbit globin mRNA (lanes 5–7) but was absent in control experiments using RNA from uninjected embryos (data not shown). A band of this size is best explained by correct splicing of the small intron in the globin gene transcript. Because 1 ng of β-globin mRNA corresponds to 3×10^9 molecules, we calculated that there were about 10^9 globin gene transcripts per embryo at gastrula stage. The protected 208-nucleotide DNA fragment was much weaker, but clearly identifiable in the autoradiograms, using RNA from hatching larvae (Fig. 4*B*, lanes 3 and 4). It appears as if the steady-state level of rabbit globin gene transcripts was roughly proportional to the number of gene copies, because hatching larvae contained much less of the injected DNA sequences than did late gastrulae (Fig. 2).

Using the same S1 nuclease mapping technique but a different probe (see *Materials and Methods*), we mapped the 5′ ends of the transcripts (Fig. 4*C*). All of the clones containing pBR322 sequences gave rise to an incorrect 5′ end, mapping approximately 306 nucleotides upstream from the *Bam*HI site (Fig. 4*C*, lanes 1, 2, and 4). However, further experiments using the circularized globin gene *Kpn* I fragment showed correct expression; the major protected DNA fragment was about 355 nucleotides long and comigrated with the fragment protected by genuine rabbit β-globin mRNA (Fig. 4*C*, lane 5). The band of full-length DNA probe present in all slots could be due to renaturation of the probe or the presence of transcripts that had initiated upstream of the *Pst* I site. Both the transcripts with the correct 5′ ends and those with the incorrect 5′ ends appeared to be polyadenylylated, because they were selectively bound to an oligo(dT)cellulose column (data not shown).

DISCUSSION

There have been several reports on the replication of foreign DNA injected into unfertilized eggs of *X. laevis* (7–12). Recently, Harland and Laskey (12) presented convincing evidence, based on density shift, for semiconservative replication of a variety of DNAs injected into unfertilized *Xenopus* eggs. In this paper we present evidence by Southern blotting for a large net increase of DNA injected into fertilized *Xenopus* eggs.

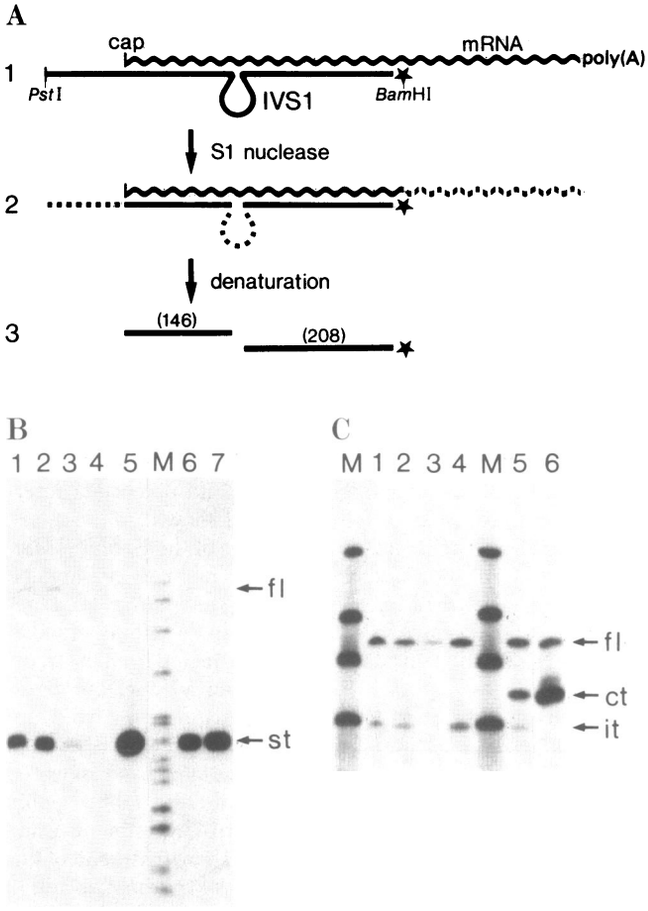

FIG. 4. Analysis of transcripts by S1 nuclease mapping. (*A* and *B*) Detection of correct splicing. End-labeled globin DNA was hybridized to unlabeled RNA from injected embryos, treated with S1 nuclease, denatured, fractionated by gel electrophoresis, and autoradiographed. The RNA was from pools of ten embryos; one-fifth was used for S1 nuclease mapping. Slots 1 and 2, hybridization to RNA from late blastulae (stage 13); slots 3 and 4, hybridization to RNA from hatching larvae (stage 35); slots 1 and 3, injection experiments with pSVK+ DNA; slots 2 and 4, injection experiment with pP+ DNA; slot 5, hybridization to total RNA from rabbit reticulocytes; slots 6 and 7, hybridization to 1 and 2.5 ng of purified rabbit β-globin mRNA, respectively; M, ^{32}P-labeled marker DNA fragments of pBR322 digested with *Hpa* II (22). fl, Full-length input DNA (579 nucleotides); st, splice terminus fragment (208 nucleotides). (*C*) Detection of the correct 5′ terminus of globin RNA. For this experiment, a globin gene clone lacking the first intron (IVS1) was used as a radioactive probe. DNA end-labeled at the *Bam*HI site was hybridized to unlabeled RNA from gastrula stage embryos and treated as above. Slot 1, RNA from embryos injected with pJKd− DNA; slot 2, injected with pSVKd+ DNA; slot 3, RNA from uninjected embryos; slot 4, injected with pSVKd− recombinant DNA; slot 5, injected with plasmid-free globin gene *Kpn* I fragment dimer circles; slot 6, rabbit β-globin mRNA; M, ^{32}P-end-labeled marker DNA fragments of pBR322 digested with *Hpa* II. fl, Full-length input DNA (453 nucleotides); ct, fragment with correct terminus, mapping 146 + 208 = 354 nucleotides upstream of the *Bam*HI site; it, incorrect terminus, about 306 nucleotides upstream of the *Bam*HI site.

We found that by 20 hr after injection the injected DNA was amplified 10 to 200 times. Linear DNA was readily ligated to large concatemeric DNA (which was also observed for other linear DNAs injected into unfertilized eggs; ref. 34; G. Galli, R. Clerc, and M. L. Birnstiel, personal communication) and was replicated in a high molecular weight form. Circular DNA seems to replicate as such, showing a considerable increase in intensity of the band of supercoiled DNA, together with the appearance of high molecular weight DNA forms (Fig. 2). It is interesting to note that pCRI plasmid DNA and the circularized rabbit globin gene fragment both were replicated in developing *Xenopus* embryos (data not shown). These DNAs originally derive from prokaryotic and eukaryotic sources, respectively. It was also observed in our laboratory that circular or linear histone genes of a sea urchin were replicated in developing *Xenopus* embryos (35). Taken together, these findings could mean that on all the injected DNAs there was at least one functional replication origin or, as suggested by the data of Harland and Laskey (12), that there is no requirement for specific replication origins in *Xenopus* early development.

The fate of the injected DNA differed from that of the host embryonic *Xenopus* DNA. While the cell number increased from 1 to about 3×10^4 cells at gastrula stage, the foreign DNA increased 10- to 200-fold, and a large fraction of it was degraded between the late gastrula and the hatching larva stage. The fact that most of the foreign DNA was degraded after gastrula stage could mean that DNA replication in late embryos becomes dependent on specific replication origins, and that only those globin genes persisted that had been integrated into host cell DNA.

At later stages of development, the number of globin genes per cell appeared to be stable: Tadpoles containing an average of 3–10 rabbit globin genes per cell developed into healthy frogs with about the same number of rabbit genes per cell. During that period, the total number of cells had increased at least 10-fold. This means that the rabbit globin genes had become a stable component of the genome in a fraction or in all of the tadpole cells. The persisting DNA in older tadpoles had the same restriction map as the circular input DNA; however, it comigrated with high molecular weight *Xenopus* DNA prior to restriction digestion, or after digestion with *Bst*EII, which cuts *Xenopus* DNA but not the input recombinant plasmid. We conclude that the globin genes persisted as head-to-tail multimers of the original input plasmid DNA. Our results thus differ from the ones of Gordon *et al.* (36), who injected recombinant plasmid DNA into fertilized mouse eggs and found that grossly rearranged forms of the input DNA persisted in a small fraction of newborn animals. A head-to-tail multimer organization is natural for many repeated genes—e.g., rRNA genes, histone genes, and tRNA genes (reviewed in ref. 37). Head-to-tail duplications and multimers were also found for viral DNA in transformed cells (38–40). The generation and persistence of head-to-tail tandem multimers after injection of circular DNA is independent evidence for the replication of input DNA in frog embryos. Multimers could arise from "rolling circle" replication, like the amplified rRNA genes in amphibian oocytes (41, 42), or from multiple rounds of "Cairns mode" replication without segregation of daughter molecules, as had been reported for mitochondrial and viral DNAs (reviewed in ref. 43). The available data do not allow us to determine whether the β-globin gene repeats were themselves part of a chromosome or minichromosome, or if they were extrachromosomal linear or circular entities.

An important finding during the course of our investigations was that the rabbit β-globin gene is transcribed in developing frog embryos. Previously, Gurdon and Brown (14) reported that after injection of gradient-purified 5S RNA genes into fertilized *Xenopus* eggs, there was synthesis of RNA that hybridized back to the injected DNA. Using the refined analysis of transcripts by S1 nuclease mapping, we found that rabbit β-globin gene transcripts with the correct 5′ end and splicing of at least one intron could be observed in *Xenopus* embryos. The correct 5′ end, corresponding to the 5′ terminus of rabbit β-globin mRNA, was found, however, only after injection of plasmid-free circular globin gene. Globin DNA linked to pBR322 (pP+, pJKd−), or to SV40 DNA in a pBR322–SV40 vector (pSVK+,

pSVKd+, pSVKd−) gave rise to transcripts with an incorrect 5′ terminus. This occurred despite the fact that in pJKd−, pSVK+, and pSVKd− there were 1.4 kb of rabbit DNA upstream of the globin gene capsite. In mammalian cells transfected with rabbit β-1 globin genes from different sources (16, 20), the same incorrect terminus was also observed either as the only terminus (44) or together with transcripts containing correct 5′ termini (ref. 45; unpublished data). This dependence of correct vs. incorrect transcription on neighboring sequences indicates a *cis*-acting effect of remote DNA sequences on gene expression (see also ref. 46). In late gastrulae (Daudin stage 13) there were 10^9 rabbit globin gene copies per embryo, which is an average of about 3×10^4 genes per cell. At the same time S1 nuclease mapping experiments indicated that there were about 10^9 molecules of rabbit globin gene transcripts per embryo. The low ratio of steady-state β-globin transcripts to β-globin genes (1:1) could be due to a reduced rate of transcription, the inability of all globin genes to be utilized as templates, or instability of the globin gene transcripts. Nevertheless, the rabbit globin gene transcripts must have represented about 1.4% of the poly(A)$^+$ RNA molecules of transformed frog embryos (47). This amount exceeds the high levels observed for several mRNA species transcribed from endogenous *Xenopus* genes (48).

It will be of great interest to see whether in tadpoles and frogs there will be tissue-specific expression of injected heterologous or homologous genes that normally are under developmental control.

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