tants (Fig. 2A). We propose that this occurs because these cell lines contain tandem duplications of plasmid sequences and that such duplications are preferred substrates for recombinational excision. To determine the optimal efficiency of the excision-rescue process, two such cell lines were fused to COS7 cells, and the amount of free plasmid in Hirt supernatants was estimated by plasmid reconstruction experiments (Fig. 2C). Lanes a, b, and c in Fig. 2C contained 1.0, 0.25, and 0.05 ng of plasmid DNA marker; lanes d and e contained 20 percent of the Hirt supernatant DNA from approximately 2×10^6 cells of two independent cell lines fused to COS7 cells. These experiments show that in approximately 50 percent of the cell lines examined, one to several nanograms of the input plasmid DNA can be isolated from approximately 10^6 fused cells. This is equivalent to the levels of excised DNA seen with bona fide SV40 transformants and is sufficient to give rise to several thousand colonies when the DNA is used to transform *E. coli* HB101 to amp^r.

Our results demonstrate that sequences directly surrounding the SV40 origin of replication are the only viral DNA sequences required in cis to mediate SV40 excision-replication after fusion to permissive simian cells. The following control experiments support this contention. No extrachromosomal plasmid DNA is detected when human cell lines transformed by pBRChTK are fused with COS7 cells or when any of the human TK$^+$ transformants are fused with permissive simian cells that do not produce SV40 T antigen. Plasmid sequences are not supplying functions important for excision and replication, since cell lines transformed by fragments of SV40 removed from the plasmid vector yield detectable replicating forms after fusion with COS7 cells (*10*). Finally, small deletions at the Bgl I site defining the origin of replication, which inactivate neither the early promoter nor T antigen binding, yield SV40 molecules capable of integration and stable transformation, but inactive for excision (*11*). This implies that it is the replication origin itself within the Eco RIIG fragment that is important for excision.

The patterns of excision products seen here (that is precise, random, or mixtures) parallel that seen with virally transformed cell lines, where the presence of tandem duplications was thought to be due to low levels of DNA replication during viral infection. It is unlikely that replication is occurring in our experiments, since the recombinant plasmid used contains no A gene. We conclude that the tandem duplications observed here, and perhaps those seen during viral transformation, occur by a recombinational mechanism. Recombination and "pekelasome" formation during DNA transfection have been reported (*12*).

The small size (0.3 kb) of the sequence required, and the ease and efficiency with which the input DNA can be recovered in *E. coli*, will make this a useful "shuttle" system for the recovery of cloned DNA sequences from mammalian cells. In addition, the simplicity of the viral sequences required may be important in minimizing the effects of the vector on the expression of a cloned gene. Since the origin is inactive in the absence of T antigen, and since the promoter shows only low levels of activity in the absence of an activator element, it is unlikely that the viral sequences will affect the expression of a linked gene. In some situations this may be preferable to other "shuttle" systems such as that provided by bovine papillomavirus (BPV) (*13*) where many of the viral coding and regulatory sequences are present.

We envision several possible applications of this "shuttle" system. A cloned gene linked to an SV40 origin could be introduced into mammalian cells, mutants selected in the phenotype conferred by that gene, and the mutated genes easily recovered by fusion with COS7 cells followed by transformation of *E. coli*. Alternatively, an SV40 origin could be introduced into a eukaryotic cloning vector so that once a gene had been identified it could be easily isolated in *E. coli*. Finally, if a gene could be introduced into the germ line of an animal (*14, 15*) its structure could be examined in many differentiated tissues by SV40-mediated excision and rescue.

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References and Notes

1. M. Botchan, W. Topp, J. Sambrook, *Cell* **9**, 269 (1976).
2. ———, *Cold Spring Harbor Symp. Quant. Biol.* **44**, 709 (1979).
3. H. Koprowski, F. C. Jensen, Z. Steplewski, *Proc. Natl. Acad. Sci. U.S.A.* **58**, 127 (1968).
4. J. F. Watkins and R. Dulbecco, *ibid.*, p. 1396. (1968).
5. W. M. Southern, *J. Mol. Biol.* **98**, 503 (1975).
6. Y. Gluzman, *Cell* **23**, 175 (1981).
7. G. Pontecorvo, *Somatic Cell Genet.* **1**, 397 (1975).
8. B. Hirt, *J. Mol. Biol.* **26**, 365 (1967).
9. D. Hanahan, D. Lane, L. Lipsich, M. Wigler, M. Botchan, *Cell* **21**, 127 (1980).
10. M. Botchan, unpublished observations.
11. Y. Gluzman, R. J. Frisque, J. Sambrook, *Cold Spring Harbor Symp. Quant. Biol.* **44**, 293 (1980).
12. M. Perucho, D. Hanahan, L. Lipsich, M. Wigler, *Nature (London)* **285**, 207 (1980); M. Perucho, D. Hanahan, M. Wigler, *Cell* **22**, 309 (1980).
13. D. DiMaio, R. Treisman, T. Maniatis, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4030 (1982).
14. F. Costantini and E. Lacy, *Nature (London)* **294**, 92 (1981).
15. J. W. Gordon and F. H. Ruddle, *Science* **214**, 1244 (1981).
16. M. Wigler, A. Pellicer, S. Silverstein, R. Axel, *Cell* **14**, 729 (1978).
17. We thank Bernard Danovitch for his expert technical assistance. This research was supported by grant MV-91 from the American Cancer Society and grant CA 30490 from the National Cancer Institute. S.E.C. was a senior fellow of the American Cancer Society, California division, and C.-P. Liu was supported by the Agri Corporation.

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Simultaneous Expression of Globin Genes for Embryonic and Adult Hemoglobins During Mammalian Ontogeny

Abstract. *The dominant hemoglobin of the adult hamster was detected in yolk-sac erythroid cells, and its identity was confirmed by peptide mapping and by analysis of relevant peptides. Both the presence and active synthesis of two embryonic hemoglobins presumed to exist only in yolk-sac erythroid cells were detected in neonatal liver and spleen. Thus the time span of expression of both embryonic and adult globin genes during mammalian ontogeny may be considerably broader than presently believed.*

Early in normal mammalian ontogeny, the most primitive erythroid cells (yolk-sac cells) of the embryo synthesize an "embryonic" set of hemoglobins, whereas in ontogenically later erythroid organs (liver, spleen, and finally bone marrow) an "adult" set of hemoglobins is made. This temporal progression in globin gene expression is a well-studied phenomenon of developmental biology. However, reports conflict on whether embryonic and adult hemoglobins can coexist in cells from the same erythropoietic organ. For example, Fantoni *et al.* (*1*) and Barker (*2*) detected only embryonic hemoglobins in yolk-sac cells and only adult hemoglobins in fetal liver cells of mice. Similar results were obtained by Bruns and Ingram (*3*) and by Beaupain *et al.* (*4*) for avian species. On the other hand, there is evidence for the coexistence of embryonic and adult he-

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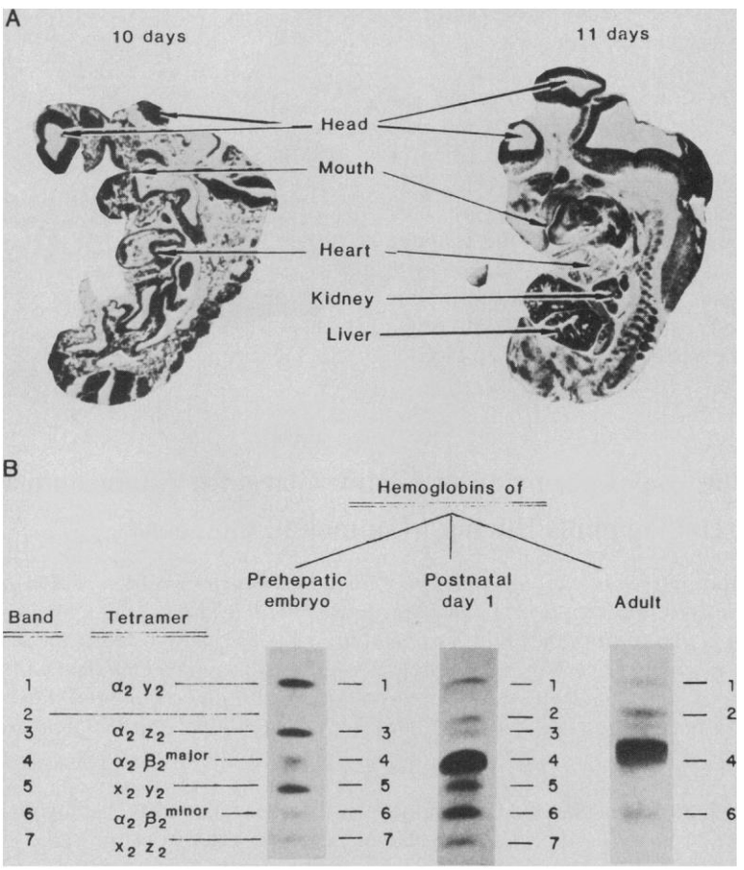
moglobin species in yolk-sac erythroid cells of hamsters (*5*) and mice (*6*) and in chick embryo erythroid cells (*7*). We report here definitive evidence, obtained by peptide mapping of isolated globin chains, for the presence of an adult hemoglobin species in hamster yolk-sac erythroid cells. We also report that embryonic globin chains are synthesized in erythroid cells from neonatal hamster liver and spleen.

Erythropoiesis in embryonic hamsters begins in the yolk-sac islands on day 7 of gestation. From day 7 to day 10 the origin of all circulating erythroid cells is the yolk sac, for the fetal liver, itself highly erythroid, does not appear until day 11 (Fig. 1A). Approximately 4 percent of the total hemoglobin in yolk-sac erythroid cells on day 10 occupies the same position in isoelectric focus patterns in polyacrylamide gel as that of the major (85 percent) hemoglobin of adult hamsters (band 4 in Fig. 1B). To determine whether the two hemoglobins are indeed indentical, we processed hamster embryos at day 10 of gestation to obtain a quantity of each of the two globin chains of the putative adult hemoglobin sufficient for peptide mapping (4 mg). Erythrocytes circulating in the embryos and those still in the blood islands were subjected to velocity sedimentation at unit gravity (*8*) with a STA-PUT cell separator (Johns Scientific) to separate yolk-sac erythroid cells (large and nucleated) from maternal erythrocytes (small and nonnucleated) (*9*). However, velocity sedimentation does not separate hamster nucleated erythroid cells of hepatic origin from yolk-sac erythroid cells. Therefore, unlike previous investigators, we procured our yolk-sac erythroid cells before the appearance of fetal liver tissue. The various hemoglobin species from these cells and from erythrocytes of adults were isolated by isoelectric focusing on polyacrylamide gel (Fig. 1B) (*10*), a technique that also eliminates carbonic anhydrase (*11*). Each band 4 hemoglobin (Fig. 1B) was eluted from the gels and cleared of nonheme proteins. Individual globins were subjected to peptide mapping (*12*). Peptide maps of band 4 α-globins from yolk-sac erythroid cells and from adult erythrocytes were identical, as were maps of the non-α-globins from the two sources. Identity was confirmed by analysis of the relevant peptides, excluding those in the trypsin-resistant core.

Having established that yolk-sac erythroid cells contain an adult-type hemoglobin in addition to the expected embryonic hemoglobins, we explored the other end of the ontogenic spectrum by searching for embryonic hemoglobins in the erythroid cells of neonatal (birth occurs at day 16) hamster spleen, liver, and peripheral blood. After cell suspensions from these three sources were incubated in nutrient medium containing a tritiated amino acid mixture, synthesis of individual globin chains—separated by electrophoresis on polyacrylamide gel contain-



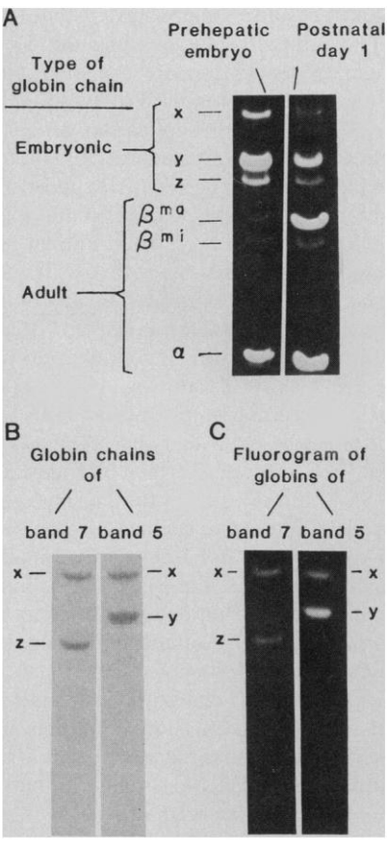


Fig. 1 (left). (A) Histological preparations of hamster embryos at approximately 10 and 11 days in gestation. The 10-day hamster embryo is 2.22 mm long and the 11-day embryo is 7.22 mm long. For purposes of comparison, however, both shown here are enlarged to the same size. Note the absence of fetal liver at 10 days. (B) Separation of hamster hemoglobin species by isoelectric focusing in polyacrylamide gel. Preparations shown are from peripheral blood before the appearance of fetal liver, from peripheral blood 1 day after birth, and from peripheral blood of the adult. Chain designations are those used for mice (*1*, *13*). The globin chains of the adult hemoglobin in bands 1 and 2 (which appears only after the fetal liver has developed) remain to be identified. Fig. 2 (right). (A) Autoradiogram of tritium-labeled globin chains from circulating yolk-sac erythroid cells at 10 days in gestation (prior to the appearance of fetal liver) and from neonatal spleen erythroid cells 1 day after birth. (B) Globin chains of hemoglobin bands 5 and 7 separated by electrophoresis in polyacrylamide gel. These bands were isolated from a mixture of yolk-sac erythroid cell hemoglobin (nonlabeled) and of spleen (1 day after birth) erythroid cell hemoglobin (labeled). The globin chains of the mixture comigrate. (C) Fluorogram of the preparation in (B). Radioactivity is strictly confined to the three embryonic globin chains.

ing urea, Triton X-100, and acetate (*13*)—was assayed by fluorography (*14*). Figure 2A shows a fluorogram comparing globins synthesized in yolk-sac erythroid cells with those synthesized in erythroid cells of the spleen 1 day after birth. In accordance with the terminology established for mice (*1, 15*), we identify the embryonic α-like globin as band x and the two embryonic β-like globins as bands y and z (*16*). The same pattern of synthesis was obtained from liver erythroid cells 1 day after birth. No embryonic chain synthesis was detected in peripheral blood cells from any neonatal sampling. Five days after birth, synthesis of two embryonic globins (x and z) was barely detectable in spleen erythroid cells only.

Further confirmation of the synthesis of embryonic globin chains in neonatal erythroid cells was provided by coelectrophoresis. Tritiated hemoglobin from erythroid cells of the spleen 1 day after birth was mixed with unlabeled hemoglobin solution from yolk-sac erythroid cells. Bands 5 and 7 (Fig. 1B) were isolated and their globins were separated by electrophoresis in polyacrylamide gel (Fig. 2B). Each band resolved only into the two globin chains characteristic of that embryonic hemoglobin (Fig. 2B), and fluorography demonstrated synthesis of each of the three globins (Fig. 2C).

There are similarities between these findings for hamsters and observations in humans. Human adult-type β-globin chains first appear at a time in gestation (6 weeks) when yolk-sac erythropoiesis is being replaced by hepatic erythropoiesis, but the earliest site of synthesis has not been defined. Trace amounts of a hemoglobin whose α-like globin is embryonic have been detected in cord erythrocytes from human neonates (*17*). Our findings demonstrate conclusively the coexistence of embryonic and adult globin gene expression over a range of developmental stages from prehepatic to early postnatal (*18*). The currently accepted time span for the normal sequence of globin gene expression during mammalian ontogeny may be too narrow.

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References and Notes

1. A. Fantoni, A. Bank, P. A. Marks, *Science* **157**, 1327 (1967).
2. J. E. Barker, *Dev. Biol.* **18**, 14 (1968).
3. G. A. P. Bruns and V. M. Ingram, *ibid.* **30**, 455 (1973).
4. D. Beaupain, C. Martin, F. Dieterlen-Lievre, *Blood* **53**, 212 (1979).
5. J. F. Bertles and T. Boussios, in *Cellular and Molecular Regulation of Hemoglobin Switching*, G. Stamatoyannopoulos and A. W. Nienhuis, Eds. (Grune & Stratton, New York, 1979), p. 237.
6. T. W. Brotherton, D. H. K. Chui, J. Gauldie, M. Patterson, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2853 (1979).
7. B. S. Chapman and A. J. Tobin, *Dev. Biol.* **69**, 375 (1979).
8. R. G. Miller and R. A. Phillips, *J. Cell Physiol.* **73**, 191 (1969). Particles (10^9 in number) were subjected to velocity sedimentation at unit gravity on a linear gradient of bovine serum albumin (1 to 2 percent) in phosphate-buffered saline (*p*H 7.3) (1600 ml of each) at 4°C. After 2 hours the peak of yolk-sac cells (rate of fall, 12.5 mm per hour) was separated from the peak of maternal erythrocytes (rate of fall, 4.5 mm per hour) by 20 mm, a distance equivalent to a gradient volume of about 1000 ml. The curves did not overlap. Fractions were pooled, the cells were washed and lysed, and clarified hemolyzates were used for further analysis.
9. Less than 0.1 percent maternal erythrocytes were detected on smears of purified yolk-sac erythroid cells stained by Wright's and Giemsa's stains and examined by light microscopy. At least 10 percent contamination by maternal erythrocytes in our purified yolk-sac cell preparations would have been required to provide the 4 percent of band 4 observed (Fig. 1B).
10. T. Boussios and J. F. Bertles, *J. Cell Sci.* **16**, 677 (1974).
11. Hemoglobin (50 μg) eluted from Sephadex G-75 superfine resin was applied to polyacrylamide gel cylinders (inner diameter, 1 mm; length, 50 mm) containing 5 percent bisacrylamide, 2 percent carrier ampholytes (*p*H 6.0 to 8.0), 0.05 percent *N,N,N',N'*-tetramethylethylenediamine, and 0.05 percent ammonium persulfate. After 20 minutes of isoelectric focusing (4°C) at 1 mA per gel tube, the hemoglobin entered the gel as a tight band (carbonic anhydrase had already migrated approximately 1 cm into the gel). The hemoglobin band was then sliced from the gel and the hemoglobins were eluted from the slice.
12. J. B. Clegg, M. A. Naughton, D. J. Weatherall, *Nature (London)* **207**, 954 (1965).
13. B. P. Alter, S. C. Goff, G. D. Efremov, M. E. Gravely, T. H. J. Huisman, *Br. J. Haematol.* **44**, 527 (1980). Hemoglobin (50 μg) freed of nonheme proteins was mixed with electrophoresis buffer consisting of 8*M* urea, 2 percent Triton X-100, 5 percent 2-mercaptoethanol, and 0.01 percent Pyronine Y. Globins were separated by electrophoresis for 4 hours at 4°C (20 mA) in a 9 percent polyacrylamide slab gel (11 by 11 by 0.1 cm), stained with Coomassie brilliant blue, and scanned at 550 nm for globin quantification. The gel was subsequently fixed, dried, and exposed to pre-flashed x-ray film (Kodak) for 2 weeks (−80°C).
14. W. M. Bonner and R. A. Laskey, *Eur. J. Biochem.* **46**, 83 (1974); R. A. Laskey and A. D. Mills, *ibid.* **56**, 335 (1975).
15. B. P. Alter and S. C. Goff, *Blood* **56**, 1100 (1980).
16. T. Boussios *et al.*, in preparation.
17. W. G. Wood, J. B. Clegg, D. J. Weatherall, *Prog. Hematol.* **10**, 43 (1977).
18. In humans (and certain other mammals), synthesis of a hemoglobin species termed "fetal" can persist at trace levels into adulthood. This is not, however, germane to our presentation, which is confined to embryonic and adult hemoglobins.
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Mammalian Muscle Acetylcholine Receptor: A Supramolecular Structure Formed by Four Related Proteins

Abstract. *The nicotinic acetylcholine receptor has been purified from fetal calf muscle. Amino terminal amino acid sequence data indicate that the mammalian receptor is formed from closely related but distinct subunits. A cytoskeletal component, actin, may be associated with the receptor.*

A human paralytic syndrome, myasthenia gravis (MG), is due to an autoimmune reaction against the nicotinic acetylcholine receptor (AcChR) (*1*). Elucidation of its structure is therefore crucial. So far, AcChR's from the electric organs of fish (*Torpedo*, *Narcine*, and *Electrophorus*) have been well characterized (*2*). Studies of mammalian AcChR have been less sophisticated; its subunit complement has been widely debated; subunit patterns containing from one to six polypeptides have been reported (*2*).

There are indications that mammalian muscle and piscine AcChR's are similar. Experimental autoimmune myasthenia gravis (EAMG) can be induced in mammals with the use of AcChR purified from electric fish (*3*), and polyclonal and monoclonal antibodies to *Torpedo* AcChR may precipitate mammalian muscle AcChR (*4, 5*).

We now report evidence that the mammalian AcChR is a complex of four homologous peptides, and that a fifth peptide that copurifies with the receptor is actin.

The AcChR was purified from fetal calf muscle (*6*) and upon sodium dodecyl sulfate (SDS) gel electrophoresis resolved into five polypeptides having molecular weights (M_r) of 42, 44, 49, 55, and 58 K (*6*) (Fig. 1A, lane 3). A sixth polypeptide of M_r 53 K was present in some preparations (*6*). The polypeptides were characterized by different methods: the presence of carbohydrate was assessed by binding of ^{125}I-labeled conconavalin A (Con A) (*7*) which consistently labeled the peptides of M_r 42, 49, and 55 K. When the polypeptide of M_r 53 K was present it was consistently labeled while those of M_r 58 K and 44 K were consistently unlabeled. The amino acid composition of the latter is similar to that of actin (*6*), including the presence of 3-methylhistidine (*8*). We confirmed that

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