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Cell Biology

Rous sarcoma virus activates embryonic globin genes in chicken fibroblasts

(globin mRNA/transformation/Cr_0t)

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ABSTRACT **Complementary DNA (cDNA) specific for chick globin mRNA sequences fails to hybridize to total RNA extracted from chicken fibroblasts. After infection by Rous sarcoma virus, RNA complementary to globin cDNA is detectable in 100–500 copies per cell. Infection of fibroblasts with the transformation defective (*td*) deletion mutant of Rous sarcoma virus leads to normal virus production, but not to host cell transformation or accumulation of RNA sequences complementary to globin cDNA. Our evidence shows that the globin genes activated by Rous sarcoma virus are those specified by embryonic chick red cells; adult-specific globin sequences were not detected.**

When Rous sarcoma virus (RSV) transforms chick fibroblasts, a large number of phenotypic changes occur in the host (1). It is not clear whether these changes involve many cellular gene products or only a few, nor whether these changes require the activation of inactive genes of the host cell, the modification of cellular gene products already being synthesized, neither, or both.

One way of measuring the genetic complexity of transformation is to see how many protein differences occur between normal and transformed cells (2). However, as usually employed, this technique can only resolve at most 200 of perhaps 10,000 cellular gene products, and usually cannot distinguish between the synthesis of new proteins and the modification of old ones. A more direct way of measuring the complexity of the cellular response to viral transformation is by DNA·RNA hybridization. In polyoma transformation of 3T3 cells (3), an analysis performed under conditions of RNA excess demonstrated a 50% increase in the amount of DNA protected by RNA sequences that were transcribed after polyoma transformation.

Although RSV has enough genetic information to code for four to five proteins, the phenotypic changes associated with RSV transformation appear to be under the control of one viral gene (4). How the product of this gene, the so-called "onc" (4) gene, could be responsible for so many seemingly unrelated changes in host function is not known. One extreme possibility is that this viral gene activates the entire host genome. As a first step toward testing this hypothesis, we ask whether a specific cellular gene, the globin gene, becomes activated after RSV transformation.

MATERIALS AND METHODS

Cell Isolation, Growth, and Virus Infection. Embryonic erythroid cells were isolated from the circulating blood of 3 to 5-day-old White Leghorn chicken embryos by vein puncture. Adult erythroid cells were isolated from the circulation of phenylhydrazine-induced anemic White Leghorn chickens. Fibroblasts were dissected from the region of the developing breast muscle of 11-day-old virus-free (SPAFAS) chicken embryos, and plated at a density of 10^6 cells per ml in 100 mm collagen-coated tissue culture dishes. Cultures were fed every day with Eagle's basic medium (GIBCO) that contained 10% fetal calf serum, 1% penicillin-streptomycin, 1% fungizone, and 2% supplemental vitamins. Cells were subcultured by removing medium from the tissue culture dish, washing twice with phosphate-buffered saline (GIBCO), and incubating in the same buffer containing 0.1% trypsin for 10 min at 37°. After incubation, cells were pipetted from the tissue culture dishes and 10% fetal calf serum was added to the suspension. Cells were then pelleted by centrifugation in a Sorvall table top centrifuge for 5 min at 1500 rpm, and plated as above. Secondary cultures were infected with the Prague strain B of RSV or the transformation-defective Prague strain of RSV (*td*) (gifts of R. Eisenman) by incubation of purified virus (5×10^6 focus-forming units) with a suspension of 10^7 fibroblasts per ml for 1 hr at 37°. The suspensions were shaken frequently, and cells were seeded at a density of 1×10^6 cells per ml in collagen-coated dishes. Addition of medium was as above. Transformation was assayed according to morphological criteria (1). All cultures were grown in the absence of dimethylsulfoxide.

Isolation of Total Cellular RNA. All extractions were performed in autoclaved glassware at room temperature; solutions were pretreated with diethylpyrocarbonate. After five subcultures, cells were washed several times with the phosphate-buffered saline and lysed by gentle homogenization in 20 volumes of 0.15 M NaCl, 0.05 M Na acetate (pH 5.1), and 0.3% sodium dodecyl sulfate. The lysate was extracted three times with equal volumes of phenol–chloroform (1:1) and several additional times with chloroform–isoamyl alcohol (24:1). The resultant aqueous phase was made 0.1 M with respect to NaCl, and the nucleic acid was precipitated overnight at −20° with 2 volumes of ethanol. The nucleic acid was recovered by centrifugation for 60 min at 11,000 rpm in the HB-4 head of a Sorvall RC-5 centrifuge. The resultant pellet was resuspended in 10 mM Tris·HCl (pH 7.4), 5 mM $MgCl_2$ and incubated at 37° for 15 min with 10 μg/ml of RNase-free DNase I (Worthington). After ethanol precipitation, the DNase digestion was repeated. After a final ethanol precipitation, the pellet was resuspended in 10 mM NaCl, 10 mM Tris·HCl (pH 7.4), and the amount of RNA was determined at OD_{260} in a Zeiss spectrophotometer (1 mg/ml of RNA = 24 OD_{260}).

Synthesis of Globin cDNA. Globin mRNA was isolated (5) from reticulocyte polyribosomes obtained from phenylhydrazine-treated, anemic White Leghorn chickens. The polyribosomal RNA was repeatedly centrifuged through isokinetic sodium dodecyl sulfate-sucrose gradients, and a single 9–10S peak was recovered. This 9–10S species was

Abbreviations: RSV, Rous sarcoma virus; Cr_0t, initial concentration of total ribonucleotide (moles of nucleotide/liter) × time (seconds); t_m, temperature at which 50% of the DNA · RNA hybrid denatures.

further purified by electrophoresis through 2.8–20% exponential, acrylamide gels. The RNA recovered from these gels was extracted with hot phenol and treated with RNase-free DNase I (Worthington). DNase was eliminated by an additional phenol extraction. This RNA directed the synthesis of adult chicken globin chains in a heterologous cell-free system (5).

The cDNA was synthesized under conditions similar to those described by Imaizumi *et al.* (6). A 100 μl sample contained 50 mM Tris·HCl (pH 8.1), 1.5 mM 2-mercaptoethanol, 10 mM KCl, 0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dTTP, 0.1 mM [^{3}H]dCTP (27 Ci/mM), 2 μg of globin mRNA, 5.0 μg of actinomycin-D, 0.6 μg of $(dT)_{12\text{-}18}$, and 2 μg of avian myeloblastosis virus polymerase. Incubation was for 1 hr at 37° and was halted by the addition of 0.1% sodium dodecyl sulfate (final concentration). The reaction mixture was passed through a column containing 2 ml of Sephadex G-50 over 0.3 ml of Chelex. The excluded cDNA was adjusted to 0.2 M NaOH and incubated at 70° for 10 min. This material was layered onto a 5–20% alkaline sucrose gradient and centrifuged at 4° for 14 hr at 40,000 rpm in a SW 50.1 rotor of a Beckman ultracentrifuge. The sedimentation value of the cDNA was about 7 S. The pooled peak was neutralized with 2 M acetic acid and precipitated overnight at −20° with 2 volumes of ethanol in the presence of 10 μg of *Escherichia coli* tRNA. The cDNA was recovered by centrifugation at 11,000 rpm in the HB-4 head of a Sorvall Rc-5 centrifuge, resuspended in 10 mM Tris·HCl (pH 7.4), and stored at −20°. The specific activity of this cDNA was approximately 10^7 cpm/μg.

Specificity of Globin cDNA Probe. The specificity of the cDNA (complementary DNA) probe for globin has previously been demonstrated (5). At 65°, 95% of the probe forms S_1 nuclease-resistant hybrids with template globin mRNA at a Cr_0t ½ of 5.6×10^{-4} (5). When reacted with total RNA isolated from chick blastoderms, fibroblasts, or muscle cells, no hybridization of the probe above background was detected, even at a Cr_0t which would permit detection of less than 10 globin mRNA molecules per cell (5). On the basis of this biological assay, we concluded that our cDNA probe is specific for globin mRNA sequences. We presume that any oligonucleotides which might be common to globin mRNA and non-globin mRNA species (7) are too short to form stable hybrids in our hybridization conditions.

Although the cDNA was prepared from adult globin mRNA, it can be used to detect the presence of globin mRNA in embryonic erythroid cells (5). This is not surprising in view of recent work (8) demonstrating that several globin chains are shared by the adult and embryonic chicken erythroid cells; in addition, many of the tryptic peptides of the unshared globin chains are identical. Approximately 70–75% of the cDNA made from adult reticulocytes is protected from nuclease digestion by globin mRNA from primitive embryonic erythroid cells (5). After formation of hybrids between the cDNA and embryonic red cell RNA and subsequent treatment with S_1 nuclease, the cDNA probe was reisolated and sedimented through a 5–20% alkaline sucrose gradient. Fifty percent of the cDNA molecules sedimented at about 7 S, indistinguishable from that of the unhybridized cDNA probe (data not shown). Embryonic red cell RNA, therefore, protects the entire length of 50% of the cDNA molecules. This supports the peptide analysis which demonstrates that embryonic erythrocytes have several globin polypeptide chains in common with the adult erythrocytes. Presumably, the protected cDNA that becomes cleaved (but not digested to trichloroacetic acid soluble material) by S_1 corresponds to the similar tryptic peptides found in globin chains not shared by adult and embryonic red cells (8). We conclude that although our cDNA is specific for adult globin mRNA sequences, it will crossreact with the homologous globin sequences synthesized by embryonic erythrocytes. Our analysis does not exclude the possibility of contamination by a non-globin, poly(A)-containing, 9–10S polysome-bound RNA that is made in red cells but not made in fibroblasts, muscle cells, or blastoderms. However, when the kinetic complexity obtained from the hybridization of purified α-chain globin mRNA with its cDNA is used as a standard (9), the Cr_0t ½ of our probe-template reaction (5.6×10^{-4}; see above) reveals a sequence complexity of about 1000 bases. This complexity corresponds to two to three globin mRNA species, consistent with the number of globin chains in the adult chicken erythrocyte (8).

RESULTS

Globin mRNA accumulates in RSV transformed fibroblasts

cDNA was made against globin mRNA from adult reticulocytes. As discussed in *Materials and Methods*, this cDNA is an excellent probe for globin mRNA isolated from embryonic red cells, inasmuch as the adult and embryonic erythrocytes have several globin chains in common (8). In addition, other globin chains represented in the cDNA population are unique to the adult cells. As shown in Fig. 1, about 94% of the cDNA probe is protected from S_1 nuclease digestion by RNA isolated from adult reticulocytes, whereas approximately 71% of the probe is protected by RNA isolated from embryonic red cells. Addition of adult RNA after the embryonic RNA reaches saturation results in a new saturation plateau of about 94% (data not shown). The difference in Cr_0t ½ of these reactions indicates that the concentration of globin sequences is lower in embryonic cells. This probably reflects the fact that our embryonic erythroid population consists mainly of erythroblasts, whereas the adult population consists mainly of reticulocytes. the difference in saturation levels reflects the fact that about 23% of the cDNA population contains sequences found only in adult globin mRNA. When reacted with total RNA isolated from uninfected fibroblasts, no protection of the globin cDNA is detected (Fig. 1). At the Cr_0t to which this experiment was carried, (log Cr_0t = 4), we would be able to detect a concentration of globin mRNA 10^{-5} to 10^{-6} of that present in erythroid cells. Thus, uninfected chicken fibroblasts do not contain a detectable steady-state level of globin mRNA.

When annealed with total RNA from RSV infected fibroblasts, over 70% of the globin cDNA is protected at saturation (Fig. 1). The Cr_0t ½ of this reaction indicates that RSV infected fibroblasts contain about 10^{-2} to 10^{-3} of the concentration of globin mRNA found in chicken erythroid cells. To determine whether the binding of the probe was a function of globin sequences contained in the viral genome, we reacted the probe with purified RSV RNA. As indicated in Fig. 1, no globin mRNA sequences were detected in the viral RNA. Thus, infection with RSV renders detectable in fibroblasts globin mRNA sequences which were not detectable before infection. As a preliminary approach to the question of whether globin genes become activated in all cells or in only a small number of cells in the population, we used the benzidine reaction as a cytological stain for hemoglobin. In over 1000 cells examined in each of several cul-

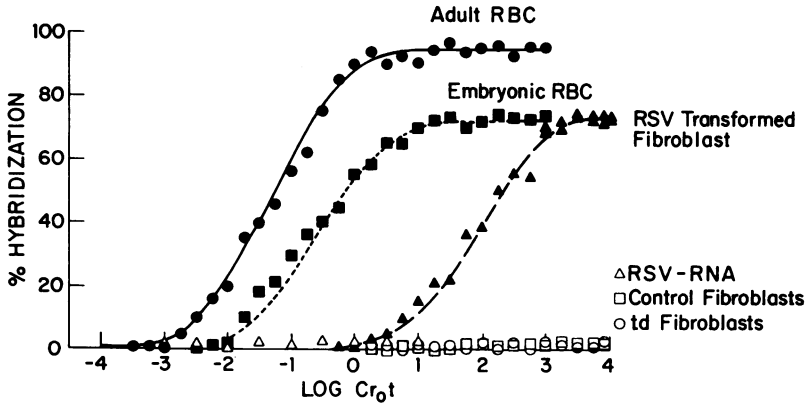

FIG. 1. Hybridization kinetics of Hb-specific cDNA with various RNA populations. Hybridizations were conducted under conditions in which RNA was in excess. RNA samples in 0.3 M NaCl, 50 mM Tris·HCl (pH 7.4), and 0.1% sodium dodecyl sulfate were denatured by heating at 100° for 5 min and then annealed at 65° with 1000 cpm of [^{3}H]deoxycytidine-labeled globin cDNA per 5 μl of reaction mixture. Hybridizations were conducted in polypropylene tubes overlayed with paraffin oil. RNA concentrations and times of incubation ranged from 10^{-3} μg/ml and 1.8 min to 10 mg/ml and 94.5 hr. At appropriate times, 10 μl aliquots of the reaction mixtures were pipetted into 400 μl of 30 mM Na acetate (pH 4.5), 0.15 M NaCl, 1 mM $ZnSO_4$, and 10 μg of denatured salmon sperm DNA; 200 μl of the above were immediately precipitated with trichloroacetic acid, and the other half was incubated with partially purified S_1 nuclease (a gift from V. Vogt) at 45° for 40 min. Percentage of hybridization was determined by comparison of the trichloroacetic acid precipitable radioactivity remaining after S_1 digestion to trichloroacetic acid precipitable radioactivity in the undigested samples. S_1 background (cpm remaining after S_1 digestion of self-annealed cDNA) was subtracted from each hybridization determination. Background ranged from 2 to 6%. The percentage of hybridized cDNA is plotted according to Britten and Kohne (16); Cr_0t = mol of ribonucleotide × sec/liter. ●, Total RNA from adult red blood cells (RBC); ■, total RNA from embryonic red blood cells; ▲, total RNA from RSV transformed embryonic chick fibroblasts; ○, total RNA from embryonic chicken fibroblasts infected with transformation defective RSV; □, total RNA from virus-free chick fibroblasts; △, RSV-RNA.

tures of control and RSV transformed fibroblasts, we detected no benzidine positive cells. The more sensitive techniques of cloning and *in situ* hybridization may resolve this question.

Is the appearance of globin sequences in infected fibroblasts a function of viral infection and replication *per se*, or is this phenomenon specifically correlated with the transforming properties of RSV? To test this, we reacted the probe with total RNA from cells infected with a transformation defective (*td*) RSV. The genome of this virus lacks the "onc" gene (4), but contains those genes necessary for host cell infection and viral production. As Fig. 1 illustrates, no hybridization of the probe is detected when the cDNA is reacted with total RNA from *td*-RSV infected cells, even though the mutant replicates and produces normal numbers of virus particles (Fig. 2). We conclude that the detection of globin mRNA in RSV transformed cells is a function of the transformed state rather than a manifestation of viral infection and replication. Finally, we have not been able to detect globin polypeptide chains in infected cells as analyzed on 15% sodium dodecyl sulfate acrylamide gels. However, since this type of analysis is not very sensitive, an immunological approach may be better.

Absence of those sequences unique to adult globin mRNA

Fig. 1 shows that RNA obtained from RSV infected fibroblasts saturates about 71% of the cDNA probe. Similarly, RNA from embryonic red cells saturates about 71% of the cDNA probe, whereas RNA from adult red cells saturates about 94% of the probe. As discussed earlier, the difference in saturation between embryonic and adult red cell RNA represents sequences in the cDNA population that are unique to adult globin mRNA. The similar saturation values between RNA from RSV infected fibroblasts and RNA from embryonic red cells led us to question whether only embryonic globin sequences were activated in RSV transformed fibroblasts. It is very difficult to accumulate enough pure globin mRNA from embryonic cells for the preparation of an embryonic cDNA probe. Consequently, we approached this question by adding embryonic red cell RNA to a hybridization mixture that contained RNA from RSV transformed fibroblasts as well as the labeled cDNA probe. If RSV activates adult-specific as well as embryonic globin mRNAs, then in these hybridization conditions more than 71% and possibly as much as 94% of the probe should be saturated. If, however, RSV activates only the embryonic globin mRNAs, then only 71% of the probe should be saturated. Fig. 3 shows that 71% of the probe was saturated. This is consistent with the hypothesis that those globin sequences that are unique to the adult are not activated during RSV transformation of chick fibroblasts. These observations also imply that several embryonic globin mRNAs may be activated. This could correspond to about four to five different polypeptide chains (8). Finally, the reason for the activation of only the embryonic globin genes is not known; at present we cannot rule out the possibility that this is a consequence of using embryonic fibroblasts in our experiments.

Stability of hybrids

To determine the stability of the hybrids formed between RNA from RSV infected fibroblasts and the globin cDNA, we heated the hybrid to the specified temperature for 10 min, cooled to 0°, and then treated with S_1 nuclease as described in the legend to Fig. 4. The hybrid between globin cDNA and either adult red cell RNA, embryonic red cell RNA, or RSV transformed fibroblast RNA melts with a sharp transition and a t_m of about 83° (Fig. 4). The same t_m is observed when the hybrid between the template adult globin mRNA and cDNA is denatured. These experiments indicate that the hybrids formed during the reannealing reactions shown in Fig. 1 are very stable ones.

DISCUSSION

The experiments reported here demonstrate that globin mRNA is not detectable in normal, uninfected chick fibroblasts; however, upon transformation by RSV, chick fibro-

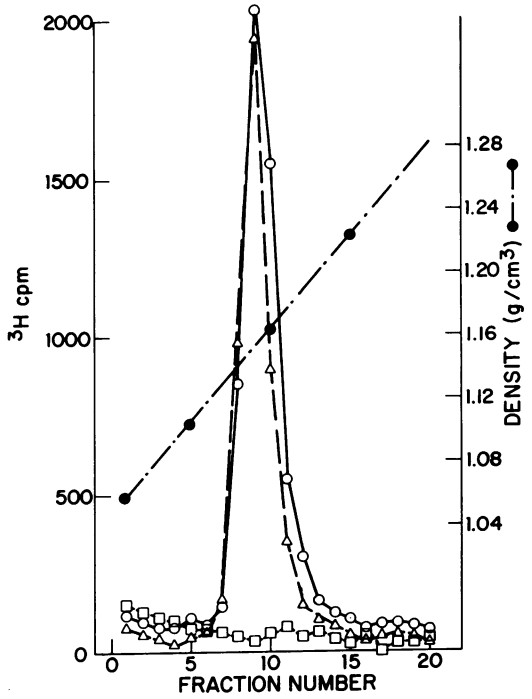

FIG. 2. Equilibrium density gradient sedimentation of virus particles. After five subcultures [^{3}H]uridine (20 μCi/ml) was added to the medium of RSV transformed fibroblasts, fibroblasts infected with transformation defective RSV (*td*), and control uninfected fibroblasts. Ten milliliters of medium was collected from the various cultures and centrifuged for 10 min at 10,000 rpm in the HB-4 head of a Sorvall RC-5 centrifuge in order to remove cells and cellular debris. The supernatant was then centrifuged for 30 min at 30,000 rpm in an SW 50.1 rotor in a Beckman ultracentrifuge. The 30,000 rpm pellet was washed several times with phosphate-buffered saline and resuspended in 200 μl of 5% sucrose in 0.01 M Tris·HCl (pH 7.4), 0.1 M NaCl, and 1 mM EDTA; 75 μl of this suspension was then layered onto a 15–60% sucrose gradient in the above suspension buffer and centrifuged for 60 min at 50,000 rpm in a SW 60 rotor. Fractions were collected from the top of the gradient with a Buchler Auto-Densiflow apparatus. Density was calculated from the refractive index. □, Control fibroblasts; ○, td infected fibroblasts; △, RSV transformed fibroblasts; ●, density (g/cm^3). For cultures infected with RSV or RSV(td), roughly equal amounts of radioactivity were incorporated into virions.

blasts are stimulated to accumulate embryonic globin RNA sequences. We shall assume that this reflects the activation of these genes, although we cannot rule out the possibility that transformation leads to the stabilization of globin RNA which is normally synthesized by fibroblasts, but is usually degraded very rapidly. In contrast, the successful infection and replication of a transformation defective RSV mutant carrying a deletion of the "onc" gene resulted in no activation of the globin genes. Thus, there appears to be a very strict correlation between the presence of the "onc" gene, host cell transformation, and the activation of embryonic globin gene transcription. Clearly, this is paradoxical because it is not at all obvious how globin gene transcription could be involved in transformation, although the presence of Hb mRNA sequences in cells transformed by the Friend virus (10) may be a precedent for this type of effect.

As discussed above and by others (11, 12), some form of generalized activation of host functions is a very reasonable strategy for transforming viruses. The problem that use of RSV confronts is how to alter a large number of cellular activities (1, 2, 13–15) given the restraints imposed by the small amount of genetic material contained in the "onc"

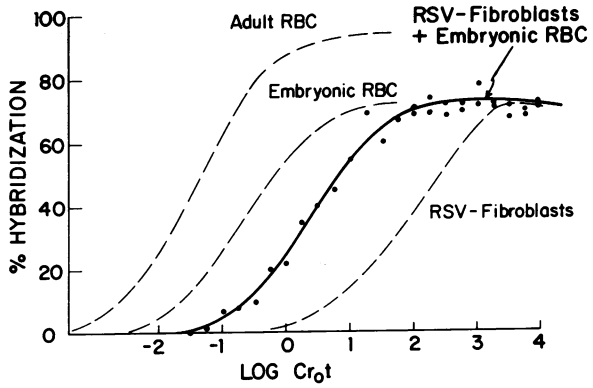

FIG. 3. Kinetics of annealing globin cDNA and an RNA mixture containing RNA from RSV-transformed fibroblasts and embryonic erythroid RNA. RNA isolation, hybridization conditions, and analysis of hybrid formation by S_1 digestion are described in Fig. 1. The RNA mixture contained 1 part total RNA from embryonic erythroid cells and 9 parts total RNA from RSV transformed fibroblasts (●). RNA concentrations and times of incubation ranged from 0.1 mg/ml and 1.8 min to 10 mg/ml and 75 hr. The dashed lines are curves taken directly from Fig. 1, showing the hybridization kinetics for adult red blood cells, embryonic red cells, and RSV transformed fibroblasts.

gene. To do this, the virus may be usurping some general cellular program; consequently, the activation of the globin genes would be symptomatic of this event. However, the failure to detect adult-specific globin RNA sequences clearly indicates that the virus does not indiscriminately activate all host cell genes.

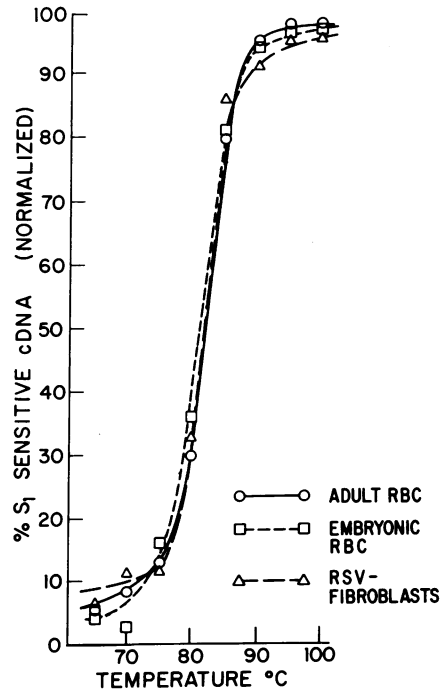

FIG. 4. S_1 melting curves of cDNA hybridized with globin RNA from adult erythroid cells, embryonic erythroid cells, and RSV transformed fibroblasts. Total RNA from adult red blood cells (RBC) (○), embryonic red blood cells (□), and RSV transformed fibroblasts (△) was annealed with globin cDNA to saturation. Aliquots (10 μl) of the hybridization mixtures were heated to the indicated temperatures for 10 min and assayed for hybrid formation by digestion with S_1 nuclease as described in Fig. 1. The curves are normalized to the respective saturation values obtained after reannealing at 65°.

Note Added in Proof. Consistent with the finding that the adult-specific globin mRNA is not activated by RSV, we have also found that RNA complementary to another adult-specific cDNA—that for ovalbumin —is also not present in RSV transformed fibroblasts. Finally, most of the activated embryonic globin RNA is found on free polysomes after RSV transformation.

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