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Activation of cellular genes by avian RNA tumor viruses

(RNA complexity/globin activation/avian erythroblastosis virus/Rous sarcoma virus)

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ABSTRACT **We demonstrated previously that chicken embryo fibroblasts accumulate approximately 100 copies of embryonic globin RNA after transformation by Rous sarcoma virus. Here we demonstrate that the globin gene in chicken embryo fibroblasts is activated by infection with two other oncogenic retroviruses, avian erythroblastosis virus and strain MC-29 of avian myeloblastosis virus, which contain transforming genes unrelated in nucleotide sequence content to each other or to the Rous sarcoma virus *src* gene. In addition, we have measured the genetic complexity of transformation by using established techniques for determining the number of different RNA sequences in specific populations of cells. Our results indicate that transformation of chicken embryo fibroblasts by Rous sarcoma virus results in the accumulation of RNA from approximately 1000 average-sized new transcription units.**

A specific viral gene, the *src* gene, is required for the initiation and maintenance of fibroblast transformation by Rous sarcoma virus (RSV) (1, 2). Some of the phenotypic changes produced in the host cell by the product of this *src* gene are well characterized (3). Other avian retroviruses can also produce a transformed phenotype in the appropriate host cell. For example, "defective" avian leukemia viruses, which require a helper virus for replication, produce acute erythroid and myeloid leukemias *in vivo* (4). Although the prototype of these defective viruses, avian myeloblastosis virus (AMV), typically transforms myeloblasts, other viral strains are capable of transforming various other cell types. Strain MC-29, which usually produces myelocytomatosis *in vivo* (5), is also capable of transforming kidney and liver cells *in vivo* (6) as well as fibroblasts *in vitro* (7). Another defective virus, avian erythroblastosis virus (AEV), transforms erythroblasts and other bone marrow cells, as well as fibroblasts, *in vivo* and *in vitro* (8, 9). Recent experiments have demonstrated that AEV-transformed fibroblasts express most of the known characteristics associated with RSV-transformed fibroblasts; MC-29-transformed fibroblasts express only a subset of these (10).

Thus, although these defective viruses do not contain nucleotide sequences related to the *src* gene of RSV (11), they are capable of producing a transformed phenotype in the appropriate target cell. Presumably, these viruses contain their own transforming genes, which may confer some specificity in terms of target cells, as well as act via a common pathway in the production of the transformed phenotype.

It is unclear at present whether the apparently large number of phenotypic changes occurring in the host cell upon viral transformation involve many cellular gene products or only a few. In addition, it is not known if these changes require the activation or inactivation of host genes, the modification of host gene products already being synthesized, neither, or both.

One way of measuring the genetic complexity of transformation is to determine how many protein differences occur between normal and transformed cells (12, 13). However, as usually used this technique can only resolve several hundred to a thousand of perhaps ten thousand cellular gene products and usually cannot distinguish between the synthesis of new proteins and the modification of old ones. A different way of measuring the complexity of the cellular response to viral transformation is by DNA·RNA hybridization. In the case of polyoma transformation, an analysis performed under conditions of RNA excess demonstrated a 50% increase in the amount of DNA protected by stable RNA sequences that were present after polyoma transformation (14); however, using different procedures, other laboratories have found few differences (15, 16).

Although RSV has enough genetic information to code for four or five proteins, the phenotypic changes associated with RSV transformation appear to be under the control of one viral gene (1, 2). How the product of this gene, the so-called *src* gene, could be responsible for so many seemingly unrelated changes in host function is not known. In regard to how the *src* gene could produce so many effects in the transformed host cell, we thought that all or many cellular genes might be activated. Several years ago, we attempted to test this hypothesis by determining whether some specific gene products, not normally found in chicken embryo fibroblasts (CEF), were present in transformed CEF. These experiments (17) showed that, whereas uninfected chicken fibroblasts and fibroblasts infected with the transformation defective (td) deletion mutant of RSV contain no detectable globin RNA sequences, the same cells transformed by RSV contain approximately 100 copies of globin RNA per cell. Further analysis demonstrated that the globin genes "activated" by RSV were those transcribed by the embryonic chicken erythroblast; adult-specific globin sequences were not detected, nor were ovalbumin RNA sequences. A similar observation concerning the presence of globin RNA in human leukemic cells has recently been made (18). Even though the activation of embryonic globin genes supports our original hypothesis to some extent, our failure to detect the activation of genes coding for adult globin or the activation of genes coding for ovalbumin clearly rules out a generalized transcriptional activation.

In the following experiments, we extended our observations concerning the activation of the globin genes by examining the globin RNA content of fibroblasts transformed by different oncogenic viral vectors. We also attempted to measure the genetic complexity of transformation by using established techniques for determining the number of different nuclear RNA sequences present in specific populations of cells. Finally, we have demonstrated that, after transformation, the accu-

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Abbreviations: RSV, Rous sarcoma virus; AMV, avian myeloblastosis virus; AEV, avian erythroblastosis virus; CEF, chicken embryo fibroblasts; td, transformation defective; RSV-CEF nDNA, DNA complementary to nuclear RNA from RSV-transformed CEF.

mulation of RNA from as many as 1000 new transcription units can be detected.

MATERIALS AND METHODS

Cells and Viruses. Fibroblasts were dissected from the region of the developing breast muscle of 11-day-old virus-free (H and N) chicken embryos and grown as described (17). Secondary cultures were infected with the Prague strain C of RSV, the transformation-defective Prague strain C of RSV (td RSV), AMV, AEV, or MC-29 virus as described (17). All viruses were the generous gifts of Maxine Linial; AMV-transformed myeloblasts were kindly provided by Joseph Beard. All cells were cultured in plastic tissue culture dishes or on roller bottles according to described methods (19) and were harvested in logarithmic phase.

RNA Isolation. Nuclei were isolated from CEF as described (20). Cells were washed twice with phosphate-buffered saline, and nuclei were isolated by suspension of cells in 0.01 M Tris·HCl, pH 7.4/0.01 M NaCl/3 mM $MgCl_2$ (reticulocyte standard buffer) containing 0.25% Nonidet P-40.

All RNA extractions were performed in autoclaved glassware at room temperature; solutions were pretreated with diethylpyrocarbonate. Cells and nuclei were prepared and washed as above and then lysed by gentle homogenization in 20 vol of 0.15 M NaCl/0.05 M Na acetate, pH 5.1/0.3% $NaDodSO_4$. The lysate was extracted three times with equal volumes of phenol/chloroform 1:1 (vol/vol), and several additional times with chloroform/isoamyl alcohol, 24:1 (vol/vol). The resultant aqueous phase was made 0.1 M with respect to NaCl, and the nucleic acid was precipitated overnight at −20°C with 2 vol 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 7.5/5 mM $MgCl_2$ and incubated at 37°C for 15 min with 10 μg of iodoacetate-treated RNase-free DNase I (Worthington) per ml. 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 from absorbance at 260 nM in a Zeiss spectrophotometer (1 mg of RNA per ml = 24 A_{260} units).

Synthesis of cDNA. Chicken globin mRNA was isolated as described (21). Ovalbumin mRNA was a kind gift of P. Thomas. cDNA was synthesized under conditions similar to those described (17, 21). A 100-μl reaction mixture contained 50 mM Tris·HCl (pH 8.1), 10 mM $MgCl_2$, 10 μg of actinomycin D, 5 mM dithiothreitol, 0.6 mg of $(dT)_{12-18}$, 0.5 mM dGTP, 0.5 mM TTP, 0.5 mM dATP, 5 nM [^{32}P]dCTP (11.1×10^{12} becquerels/mmol), 5 μg of RNA, and 40 μl of avian myeloblastosis virus polymerase. Incubation was for 4 hr at 37°C, and the reaction was stopped by addition of $NaDodSO_4$ to 0.1%. The reaction mixture was centrifuged through a 1-ml column of packed Sephadex G-50 layered over sterile sea sand. The excluded cDNA was adjusted to 0.3 M NaOH and incubated at 65°C for 0.5 hr. The mixture was then neutralized, and the cDNA was precipitated with 2.5 vol of ethanol overnight at −20°C. For most experiments, the globin cDNA had a specific activity of approximately $2–4 \times 10^8$ cpm/μg; the ovalbumin cDNA was used at specific activity of about $4–6 \times 10^7$ cpm/μg. The specificity of the globin cDNA has been described in detail (17).

Hybridizations in Cellular RNA Excess. RNA samples in 0.3 M NaCl/50 mM Tris·HCl, pH 7.4/0.1% $NaDodSO_4$ were denaturated by heating at 100°C for 5 min and then hybridized at 65°C. Hybridizations were conducted in polypropylene tubes overlaid with mineral oil. RNA concentrations and times of incubation ranged from ng/ml and 1.8 min to 15 mg/ml and 155 hr. At appropriate times, 2-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 $ZnSO_4$ containing 10 μg of denatured salmon sperm DNA; 200 μl of this was immediately precipitated with trichloroacetic acid, and the other 200 μl was incubated with partially purified nuclease S1 (Bethesda Research Laboratories, Rockville, MD) at 45°C for 40 min. Percentage of hybridization was determined by comparison to the trichloroacetic acid-precipitable radioactivity in the undigested samples. Nuclease S1 background (cpm remaining after digestion of self-hybridized cDNA) was subtracted in each hybridization determination. Background ranged from 2% to 6%. The percentage of hybridized cDNA is plotted as described (17, 21). C_rt = initial concentration of RNA (mol of ribonucleotide per liter) × time (sec).

Unlabeled DNA was isolated from chicken erythrocytes as described (20) and labeled *in vitro* by nick-translation (21) with [^{3}H]dCTP and [^{3}H]TTP to a specific activity of about 2×10^7 cpm/μg. The labeled DNA was then sheared to a mean single-strand length of 250 nucleotides. Single-copy DNA was isolated by hydroxylapatite chromatography as described by Galau *et al.* (21).

RESULTS

Activation of Globin Genes by AEV and MC-29. In attempting to determine whether there is a unique subset of genes commonly activated by transformation, we assayed for the presence of globin RNA in fibroblasts transformed by AEV and MC-29, two viruses that are capable of transforming fibroblasts but contain transforming genes unrelated (in terms of nucleotide sequence) to RSV or to each other (11). Fibroblasts transformed by these two viruses contained significant amounts of globin RNA, whereas control fibroblasts contained no detectable globin RNA (Fig. 1). As controls, ovalbumin RNA was not detected in either control cells or AEV- or MC-29-transformed cells (not shown). Thus, each of three transforming viruses (RSV, AEV, and MC-29) with distinct transforming genes is capable of activating a specific cellular gene in transformed fibroblasts. We assume that the appearance of globin RNA in our transformed cultures reflects the activation of the globin genes in all of the cells in the population because the globin chromatin

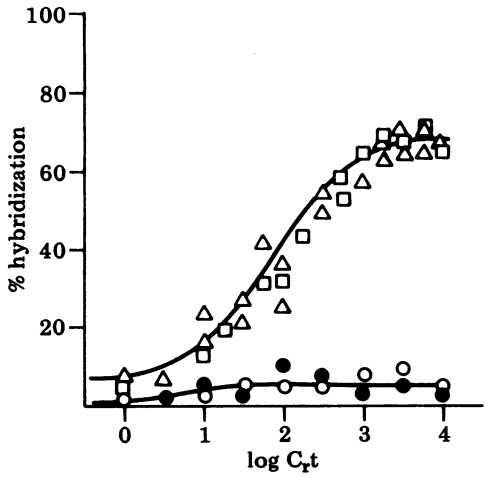

FIG. 1. Accumulation of globin RNA after transformation of CEF by AEV and MC-29. Total RNA from CEF transformed by AEV (□) or MC-29 (△) was hybridized to globin cDNA. Similar reactions were carried out with control CEF (○) and with RNA from AMV-transformed myeloblasts (●). Each reaction mixture contained total RNA at 15 mg/ml in a volume of 25 μl. Input globin cDNA was 10,000 cpm, and the probe had a specific activity of 4×10^8 cpm/μg.

in these cells acquires a DNase-sensitive conformation (20) after RSV transformation (unpublished data).

In contrast, infection of CEF with AMV, another RNA tumor virus, failed to result in the accumulation of globin RNA sequences (data not shown). Although this virus is capable of producing a transformed phenotype in certain leukocyte precursors, AMV infection of fibroblasts does not result in the formation of sarcomas *in vivo* or transformation *in vitro* (5). To determine if AMV activates the globin gene in the virus' principal target cell, we assayed AMV-transformed myeloblasts for the presence of globin RNA. No globin sequences were detectable in these cells (Fig. 1). Thus, globin gene activation may be associated with the ability of an RNA tumor virus to transform fibroblasts or produce sarcomas rather than with the ability of the virus to transform a specific target cell.

The Genetic Complexity of Transformation. Although the activation of the globin genes may provide a useful approach to the study of the effects of transformation and the future analysis of how these genes are activated, it is likely that hemoglobin is not required for the maintenance of transformation or for viral production. Thus, the activation of the embryonic globin genes might reflect the activation of some essential cellular program required by the virus for transformation. Therefore, we attempted to determine if a specific subset of genes is activated by RSV transformation of fibroblasts.

Nuclear RNA was isolated from transformed and nontransformed cells (nontransformed cells are CEF from embryonic breast muscle infected in culture with td RSV; transformed cells are from parallel cultures of CEF after infection and transformation by wild-type RSV Prague strain C). When vast excesses of these nuclear RNA preparations were hybridized with tracer amounts of labeled single-copy chicken DNA, approximately 10–11% of the single-copy DNA was hybridized at saturation by nuclear RNA from nontransformed CEF and approximately 13–15% of the same DNA was protected from nuclease S1 by nuclear RNA from RSV-transformed fibroblasts (Fig. 2). If it is assumed that the average transcription unit is about 10 times larger than its corresponding mRNA, the observed differences in saturation represent the appearance of about 1000 new transcription units in the transformed cell population. The kinetics of the transition (see also Fig. 3) would also suggest that these additional sequences are present at roughly the same level as the majority of nuclear RNA molecules and, hence, it is likely that each of these new sequences is present in every transformed cell.

To verify these results further, as well as to attempt to isolate those genes specifically activated by RSV transformation, we hybridized preparative quantities of the transformed cell nuclear RNA and nick-translated, high-labeled single copy DNA. After saturation was achieved, the hybrids were purified by hydroxylapatite chromatography and the RNA was degraded by treatment with alkali. Those [^{3}H]DNA sequences complementary to transformed cell nuclear RNA (RSV-CEF nDNA) were then isolated by precipitation with ethanol. This RSV-CEF nDNA hybridized much more efficiently to RNA from transformed cells (Fig. 3*A*). This again shows that a considerable number of new RNA sequences accumulated after transformation by RSV.

In attempting to isolate those sequences uniquely transcribed in the transformed cell, we hybridized a vast excess of nuclear RNA from nontransformed td RSV-infected CEF with the RSV-CEF [^{3}H]nDNA. At saturation of this hybridization reaction, the reaction mixture was passed over hydroxylapatite and the single-stranded DNA was isolated. This DNA represents those sequences present in RSV-transformed fibroblasts but not in fibroblasts infected with the RSV mutant deleted in the *sarc* gene. This "probe" was then purified by degrading the RNA with alkali and precipitating the DNA with ethanol. In order to ensure the purity of the probe, the DNA was hybridized twice more to td RSV-infected CEF RNA and each time the single-stranded DNA was isolated. When this probe was hybridized to nuclear RNA from RSV-transformed cells, about 50% hybridization was achieved (in theory, we would expect 100% hybridization, but we consistently find that the probe loses hybridization efficiency after repeated hybridization and treatment with alkali) (Fig. 3*B*). In contrast, less than 5% of these sequences were hybridized by the nuclear RNA from control cells. Thus, our results indicate that there is a unique

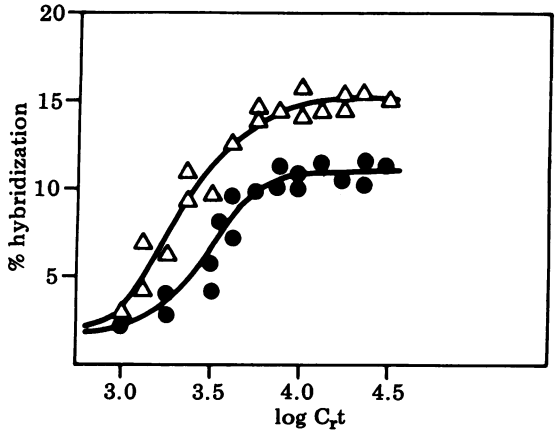

FIG. 2. Increase in number of stable transcription products in nuclear RNA of CEF upon transformation by RSV. Nuclear RNA from td RSV-infected CEF (●) hybridized 10–11% of ^{3}H-labeled unique sequence chicken DNA, whereas nuclear RNA from RSV-infected CEF (△) hybridized 13–15%. Each reaction mixture contained RNA at 15 mg/ml in a volume of 50 μl. Input DNA was 10^5 cpm. The ^{3}H-labeled unique sequence DNA used as tracer hybridized to 89% with log $C_0t_{1/2}$ 2.8 to total chicken DNA sheared to 450 base pairs. Thus, based upon saturation values of 11% and 14% and assuming assymetric transcription, td RSV-infected CEF nuclear RNA and RSV-infected CEF nuclear RNA represent approximately 25% and 32% of the unique sequence complexity of the chicken genome, respectively.

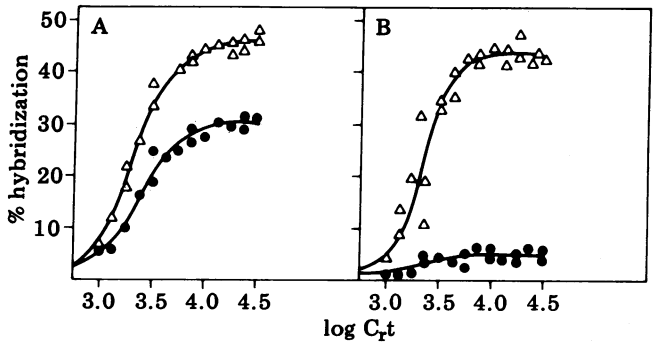

FIG. 3. Isolation of those genes uniquely transcribed in RSV-transformed CEF. (*A*) Approximately 50% of the ^{3}H-labeled RSV-CEF nDNA was complementary to RSV-transformed CEF nuclear RNA (△), whereas approximately 30% was complementary to td RSV-transformed CEF nuclear RNA (●). Hybridization reactions reached saturation at 50% (rather than 100%) due to the loss of hybridizable cpm of DNA during the RSV-CEF nDNA isolation procedures. Hybridization conditions were as described in Fig. 2. (*B*) At saturation, the reaction mixture containing td RSV-CEF nuclear RNA and ^{3}H-labeled RSV-CEF nDNA was passed over hydroxylapatite and the single-stranded, nonhybridized subset of the RSV-CEF nDNA was isolated. This fraction contained genes uniquely transcribed in the RSV-transformed CEF. It was hybridized to nuclear RNA from RSV-transformed or td RSV-transformed CEF.

subset of genes activated in CEF by RSV, and an RSV "transformation-specific" DNA probe can be readily isolated. It remains to be determined whether such transformation-specific sequences are common to cells transformed by other oncogenic agents, as well as whether these sequences are present in the cytoplasmic fraction of RSV-transformed CEF. Preliminary studies in our laboratory indicate that, like the globin gene, most of the sequences activated by RSV are also activated in MC-29- and AEV-transformed fibroblasts. Moreover, approximately 10% of the RSV transformation-specific nuclear RNA sequences can be detected in the cytoplasm of RSV-transformed fibroblasts.

DISCUSSION

From two previous studies (15, 16) attempting to find differences in RNA species after transformation, it was concluded that, if such differences do occur, they are marginal. In contrast, the experiments reported here detect large differences in RNA species present after transformation. We believe that there are two main reasons that can account for the differing results. The previous studies used cDNA to poly(A)$^+$ RNA as probe; we used total single-copy chicken DNA. As a result, our analysis is not affected by RNA species present at different abundancy levels. Hence, our assay is much more sensitive. A second difference is that our "control" cells are primary cells. The previous studies used cell lines as controls, and it is possible that these cells, by virtue of being selected as lines, were already marginally transformed.

In this report we have demonstrated that a particular gene, the globin gene, is activated in chicken fibroblasts transformed by three oncogenic viruses whose transforming genes are unrelated in terms of nucleotide sequence. In addition, the ovalbumin gene is not activated in CEF by any of these viruses. In contrast, another virus (AMV) capable of transforming myeloblasts but incapable of transforming fibroblasts does not activate the globin genes in either myeloblasts or fibroblasts. Thus, globin gene activation may be symptomatic of the cellular alterations accompanying transformation of fibroblasts by oncogenic viruses but not of those changes associated with transformation of other nonfibroblastic cells.

As discussed above and by others (17, 22), some form of generalized activation of host functions (23) is a reasonable strategy for transforming viruses. The problem confronting the transforming vector is how to alter a large number of cellular activities, given the restraints imposed by the small amount of genetic material contained in the *src* gene. One way of accomplishing this alteration may be the usurption by the virus of some general cellular program; the selective activation of the embryonic globin genes might then be symptomatic of this event. Our failure to detect adult-specific globin RNA or ovalbumin RNA in RSV-transformed CEF (17) clearly indicates that the virus does not indiscriminately activate all host genes. However, our current demonstration that the activity of the *src* gene product results in the accumulation of RNA from an estimated 1000 new transcription units supports the notion that a subset of genes is activated in the transformed state.

The importance of these transformation-specific transcripts in establishing and maintaining the transformed phenotype is unclear. The transcription of these sequences could occur as a secondary and perhaps irrelevant consequence of other primary events of transformation. Indeed, the evidence that the *src* gene product is a protein kinase (23) primarily located in the cytoplasm and that many of the characteristic properties of transformation are a consequence of a cytoplasmic src protein (24) certainly suggest that new host transcription is not necessary for transformation. However, because it is still unclear in a biochemical sense what "transformation" is, it is impossible at present to determine whether transcriptional activation is a necessary prerequisite or a consequence of the transformed state.

Note Added in Proof. Screening a recombinant chicken DNA library with RSV-CEF [^{32}P]nDNA, we have been able to identify over 100 recombinants containing sequences represented in the transformation-specific probe.

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