Alteration of *in vivo* DNA synthesis in the alpha globin locus of chick embryo fibroblasts due to *in vivo* activity of Rous sarcoma virus pp60^{src}

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ABSTRACT

Globin gene transcription is activated in chick embryo fibroblasts (CEF) transformed by Rous sarcoma virus (RSV). To determine whether this activation is correlated with a change in the replication of the alpha^D globin locus we used a nuclear runoff replication assay. Density labeling of nuclei showed that replication of the alpha^D globin gene in normal and RSV-transformed CEF (RSV-CEF) is in the transcriptional direction. However, in RSV-CEF the signal from a specific restriction fragment between the alpha^D and alpha^A globin genes was lower in the replicated DNA. This decrease was selective in that the signal of this restriction fragment was not diminished in the unreplicated DNA in the same nuclei. When CEF transformed by temperature sensitive RSV were grown at the restrictive temperature for pp60^{src} activity the loss of transformed cell morphology correlated with the reappearance of the replicated alpha globin DNA fragments in their normal stoichiometry. By comparison, histone H5 gene expression was not seen in CEF or in RSV-CEF, and H5 gene replication was not perturbed by viral transformation. These results suggest that the viral pp60^{src} protein can activate transcription of the alpha^D globin gene and change the replication of alpha globin chromatin.

INTRODUCTION

Transcription factor binding near the replication origins of eucaryotic viruses can stimulate replication and transcription (1). A similar synergy may underlie the early S phase replication of transcribed sequences in complex eucaryotic chromosomes (2, 3). While several transcriptional regulatory motifs have been identified, less is known about DNA sequences governing

replication and their possible relationship to transcriptional control. The *S. cerevisiae* autonomously replicating sequence (ARS) elements contain AT-rich tracts resembling recognition sites for the TATA binding transcription factor. Selective protein binding to ARS consensus sequences (4, 5), implies that specific sequences are recognized for replication initiation.

That several murine, avian and human genes display a preferred polarity of replication suggests that replication begins nonrandomly in eucaryotic chromosomes (6–10). Identification of replication origin zones for the rodent dihydrofolate reductase [DHFR] (11–16), opsin (17) and adenosine deaminase genes (18), the avian alpha^{pi} globin gene (19, 20) and the human c-myc gene (9, 10, 21) provides direct evidence for defined initiation regions. In the transcribed immunoglobulin, histone H5, globin and c-myc loci (5–10), replication occurs predominantly in the direction of transcription, while replication appears to occur anti-transcriptionally in the hamster DHFR/2BE2121 locus (11–16). To investigate the possible link between replication and transcription, we examined the replication of the alpha^D globin locus when its transcription was induced in chick embryo fibroblasts (CEF) by Rous sarcoma virus (RSV). Integrated viral DNA was not detected within the alpha globin locus in infected cells, yet the virus could act at a distance to change the structure of the replicated DNA specifically between the alpha^D and alpha^A globin genes. RSV transformation decreased the hybridization signal of a specific restriction fragment in runoff replicated alpha globin DNA, while a corresponding decrease in the signal of this fragment was not seen in the unreplicated DNA in the same population of nuclei. In CEF infected with a temperature sensitive RSV strain (tsNY68) the loss of viral pp60^{src} protein activity *in vivo* correlated with the loss of this altered replication structure. These data show that the pattern of replication of a specific region of DNA can be reversibly altered in response to the transcriptional state or other transient properties of the region.

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MATERIALS AND METHODS

Cell culture

14 day old white Leghorn CEF were cultured in DMEM with 10% tryptose phosphate broth, 4% fetal calf serum, 2% chicken serum and 0.1% gentamycin at 35°–41°C in 5% CO₂ and 95% air. CEF (~5×10⁶ cells; passages 5–6) were infected with tsNY68 or wtRSV (Schmidt-Ruppin A) in suspension for one hour at 37°C (24, 25), plated at 2.5×10⁵ cells/25 cm² flask, and fed with fresh medium on the following day.

In vivo transcription

Radiolabeled RNA was synthesized *in vivo* using [α -³²P]-UTP (26), and hybridized with plasmid or phage DNAs immobilized on nitrocellulose (Schleicher & Schuell). Cloned DNAs [pA1 (27); pSRA-2 (28); pCA-beta-G1 (29); p-alpha^D (30); λ FC3B, λ FC40 (31); and pH4 (32)] were digested with restriction enzymes as described in Figure 1, legend.

In vivo nuclear runoff replication

Analyses were performed as described (7–9), using [α -³²P]-dCTP labeled random primed probes (33). When nuclei were digested with restriction enzyme in the replication reaction they were preincubated for 30 minutes with *Eco*R1 or *Hind*III at 50 U/ μ g DNA. In reactions without predigestion, purified DNA was digested to completion with *Eco*R1 or *Hind*III. *In vivo* replication reactions were performed at 37°C. Replicated DNA (heavy-light BrdU labeled; density 1.75–1.78 g/ml) and unreplicated DNA (light-light; unlabeled; density 1.69–1.71 g/ml) were separated by CsCl centrifugation. The histone H5 cDNA (0.86 kb) and globin DNA probes are shown in Figure 3 (34); the viral src probe was a 1.7 kb *Hinc*II/*Eco*R1 fragment from pSRA-2 (28).

Densitometry

Four to eight autoradiographic exposures of each filter were scanned using an LKB 2202 Ultrascan soft laser densitometer (7). Although overexposures of some autoradiograms were photographed to reveal faint bands, only exposures within the empirically determined (7) linear range of film response were scanned. Traces were scaled to match the response from the *in vivo* and *in vivo* reactions for direct comparison. Each replication result shown is representative of 3–5 independent experiments. Virtually identical results were obtained from duplicate DNA aliquots subjected independently to all analytical steps.

RESULTS

Characterization of chick embryo fibroblasts infected with RSV

Early passage CEF were infected with wild-type RSV or a temperature sensitive RSV strain (tsNY68) encoding a heat-labile pp60^{src} protein (35). Within one week of growth at 35°–37°C greater than 90% of the cells in the infected cultures assumed the rounded shape and refractile appearance attributed to RSV-transformed CEF (24), whereas uninfected CEF grew in smooth, regular arrays. When tsNY68 infected CEF (tsRSV-CEF) were cultured at the non-permissive temperature (41°C) the transformed morphology of the infected cells reverted to the normal CEF morphology over a period of 24–48 hours; these cells could be returned to the transformed morphology by a downshift in temperature from 41°C to 35°C (35).

Northern and slot blot analyses of RNA revealed a >100-fold increase in RNA complementary to viral src cDNA (relative to ribosomal RNAs or beta actin RNA) in RSV-CEF grown at 35°C or 41°C compared to uninfected CEF (not shown). As quantitated by DNA dot blot hybridization RSV-CEF carried an average of 3–4 viral src genes per cell (data removed). Restriction enzyme mapping detected no change in the structure of the alpha globin domain in the RSV-CEF cultures; preferential proviral integration did not occur within the boundaries of the restriction enzyme target sites used (~10–15 kb 5' or 3' to the alpha globin genes), although preferred sites for retroviral cDNA integration near open chromatin structures have been noted in other systems (36, 37).

Induction of alpha^D globin expression in RSV-CEF

The alpha^D globin genes are not detected as stable mRNA in normal CEF, but sequences complementary to globin cDNAs have been found by solution hybridization to RNA from RSV-CEF (25). To test whether the accumulation of stable globin RNA in RSV-CEF was associated with the induction of transcription radiolabeled RNA was synthesized by runoff transcription in RSV-CEF nuclei and hybridized to immobilized DNAs (Figure 1A). RSV-CEF RNA hybridized to alpha^D globin, beta globin, histone H4 and beta actin DNAs, but not to vector sequences present in mass excess on the filter. [Beta globin hybridization is not proof of transcription of this locus, as the beta globin clone used can hybridize to repetitive sequences in RNAs transcribed from other loci (29)]. In contrast, RNA synthesized *in vivo* in nuclei from uninfected CEF hybridized to beta actin, histone H4, src and fibronectin DNAs but not to alpha globin, beta globin, RSV env, or vector DNAs (Figure 1B). Northern blot analyses were also consistent with the presence of stable globin mRNA in RSV-CEF but not in uninfected CEF; histone H5 mRNA was not detected either in RNA from uninfected CEF or RSV-CEF (not shown). These results suggest that the increase in alpha globin RNA in RSV-CEF is due to the induction of transcription.

In vivo nuclear runoff replication

To determine whether RSV activation of globin transcription was associated with a change in the replication of the alpha globin locus we used nuclear runoff replication (Figure 2). Runoff replication assays have identified the human c-myc (9, 10), avian alpha^{pi} globin (20) and DHFR origins of replication (38), and have elucidated the replication polarity of the alpha globin and histone H5 loci in several cell types (7, 8, 20). Thus, this assay is a valid approach for the study of eucaryotic chromosomal DNA replication. Previous experiments in our laboratory, and others, have shown that de novo initiation of replication is at a level below the limit of detection (7–9) and that DNA synthesized in isolated nuclei is extended from forks initiated in intact cells (7–10, 38). The predominant mechanism of DNA synthesis in this system is not repair patch replication since uniformly heavy-light DNA greater than 5–15 kb in length is synthesized in the presence of BrdUTP (7). Replication of the hamster DHFR locus in a comparable *in vivo* system has been shown reflect the temporal specificity of DNA synthesis *in vivo* (38).

The relative incorporation of BrUdR into neighboring DNA restriction fragments was deduced after DNA purification, restriction enzyme digestion, neutral CsCl density gradient centrifugation, electrophoresis, Southern blotting and hybridization of the density labeled DNA. Comparison of the signals from two restriction fragments in the same gel lane compensates for artifacts during these steps. For standardization,

Figure 1. Induction of α^D globin gene expression in RSV-CEF. Radiolabeled RNA was synthesized by runoff transcription in RSV-CEF nuclei (A, B) or uninfected CEF nuclei (C, D) and hybridized to cloned DNAs. (A) Ethidium bromide stained gel of digested plasmids. The two leftmost lanes contain size markers. (B) Autoradiogram of filter transfer of gel in (A) after hybridization. Lanes a and a', pH4 *Eco* R1 digest, 1.2 kb histone H4 DNA; lanes b and b', pCA-beta-G1 *Eco* R1 digest, 6.2 kb beta globin DNA; lanes c and c', p-alpha^D *Bam*H1 digest, 1.6 kb alpha^D globin DNA; lanes d and d', pA1 *Hind*III digest, 2.0 kb beta actin cDNA; lanes e and e', pSRA-2 *Bam*H1 digest. Signals due to hybridization to the beta globin (*) and alpha^D globin (→) DNAs are indicated. (C) Ethidium bromide stained gel of digested DNAs. (D) Autoradiogram of filter transfer of gel in (C) after hybridization. Lanes a and a', pSRA-2 *Pvu*II digest; lanes b and b', pH4 *Eco* R1 digest; lanes c and c', pCA-beta-G1 *Sma*I, *Sst*I, *Hind*III digest; lanes d and d', p-alpha^D *Bam*H1 digest; lanes e and e', pA1 *Hind*III digest; lanes f and f', plasmid pSRA-2.env (pSRA2 subclone) *Eco*R1, *Bam*H1 digest, 0.65 kb RSV env gene fragment; lanes g and g', phage λFC40 *Eco*R1 digest, fibronectin gene fragments; lanes h and h', blank; lanes i and i', phage λFC3B *Eco*R1, *Hind*III digest, fibronectin gene fragments; lanes j and j', pSRA2-5.2 (pSRA2 subclone) *Sst*I, *Sal*I digest, 3.3 kb src fragment. Hybridization to FC40, FC3B, and RSV src DNA reproduce results obtained by Tyagi *et al.* (31).

the signal ratio of two restriction fragments replicated *in vivo* was normalized to the signal ratio for the same two fragments labeled for one-half cell generation with BrUdR *in vivo* and banded at the same position and density on a parallel CsCl gradient (Figure 2A).

A normalized restriction fragment signal ratio of unity indicates that neighboring restriction fragments are replicated *in vivo* with the same stoichiometry as they are *in vivo*. As in previous studies of the replication polarity of the alpha globin (7), histone H5 (8) and c-myc genes (9) the normalized signal ratio of restriction

Figure 2. *In vivo* runoff replication. (A) Density labeled control DNA was prepared from cells labeled in culture [*in vivo*] for one-half generation with BrUdR. DNA was digested with restriction enzymes and banded in parallel neutral CsCl gradients with DNA density labeled in nuclei. (B) Runoff density labeling in the absence of restriction enzyme [*in vivo* (-)]. Nuclei from an asynchronous cell culture were incubated in replication mixture containing BrdUTP. DNA was purified, digested with restriction enzymes and banded on CsCl. (C) Runoff density labeling in the presence of restriction enzyme [*in vivo* (+)]. Nuclei from an asynchronous cell culture were incubated in replication mixture containing BrdUTP and restriction enzyme (*Eco*R1, alpha globin DNA replication; *Hind*III, histone H5 DNA replication). DNA was purified and digested with a second restriction enzyme (*Bam*H1, alpha globin DNA replication; *Pst*I, histone H5 DNA replication) and banded on CsCl. Density labeled DNA was isolated from each of the gradients, electrophoresed and blot hybridized.

fragments was highly reproducible despite the potential variation between independent labeling reactions and centrifugations (cf. Figures 4–6). Events which alter the stoichiometry of replicated DNA fragments (e.g. the presence of a barrier to *in vivo* replication fork movement, degradation of replicated DNA fragments) will change the normalized ratio of restriction fragments from unity. Models predicting the effect of a replication fork barrier on the recovery of *in vivo* replicated DNA fragments have been presented (7–9). Prior experiments have confirmed that a barrier to fork movement within ~4–5 kb of the region under analysis could detectably influence the runoff replication pattern of two neighboring ~1 kb DNA fragments (7). In some of the experiments described, a restriction enzyme was added to the replication reaction to introduce such a barrier.

Replication of the alpha^D globin locus in CEF

Restriction enzyme cleavage sites near the alpha globin locus and probes complementary to the 3' region of the alpha^D globin gene are shown in Figure 3 (30, 39, 40). The replication of this region was analyzed using nuclei from asynchronously growing CEF cultures and allowed to continue DNA synthesis in the presence of BrdUTP (see Figure 2B). The data of Figure 4 show that the relative amount of density labeled 0.6 kb *Bam*H1-*Eco*R1 fragment ('0.6 kb fragment') and 1.0 kb *Eco*R1-*Bam*H1 fragment ('1.0 kb fragment') *in vivo* was closely similar to the relative amounts of these fragments density labeled *in vivo* (Figure 4, top). Therefore, this assay detected no preferential DNA loss or endogenous barrier to fork movement between these fragments.

Figure 3. Avian alpha globin and histone H5 gene loci. Black boxes, exons; stippled boxes, introns; arrows indicate the direction of transcription. (Upper) Alpha globin locus. Open rectangle denotes globin hybridization probes. (Lower) Histone H5 locus. The open rectangle denotes the H5 cDNA probe. The 2.6 and 3.2 kb fragments are generated as the result of a *Pst*I site polymorphism. The relative amounts of the 3.2 kb and 2.6 kb fragments vary between animals and between mixed CEF cultures derived from more than one animal. E, *Eco*R1; B, *Bam*H1; P, *Pst*I; H, *Hind*III. The distance between the *Hind*III sites in the histone H5 region is approximately 7.5 kb.

Similar results were obtained in previous studies of the alpha globin genes in embryonic RBCs, where the locus is in a transcribed, DNase I sensitive state, and in MSB lymphoid cells, where it is inactive (7).

To determine the direction of replication in this region *Eco*R1 was added to the runoff replication mixture to cleave between the 0.6 kb and 1.0 kb fragments and introduce an exogenous barrier to fork progression near the 3' end of the α^D gene (see Figure 2C). As a result, the recovery of the density labeled downstream 1.0 kb fragment decreased by roughly 50% relative to that of the upstream 0.6 kb fragment (Figure 4, bottom). The decreased recovery of the density labeled 1.0 kb fragment suggests that the 0.6 kb fragment is located on the side of an *Eco*R1 generated barrier which is closer to the replication origin, and therefore that the CEF α^D genes are preferentially replicated in the transcriptional direction.

The 1.1 kb band seen in the lane containing DNA replicated *in vivo* in the presence of *Eco*R1 (*in vivo* (+)) reflects cross-hybridization to a partial digestion product of the α^{pi} gene (40) and does not bear on this experiment. Incomplete digestion of the 1.6 kb *Bam*H1-*Bam*H1 fragment, which comprises the 0.6 kb and 1.0 kb fragments, also would not alter the interpretation that this region is replicated in the transcriptional direction, because both the 0.6 kb and 1.0 kb fragments derive from the same *Eco*R1 cleavage; thus, partial digestion would not change the ratio of these fragments. Replication of the alpha globin locus in the transcriptional direction has also been observed in 5 day RBCs and MSB cells (7).

Consistent with these results, we have confirmed that a replication origin active in embryonic chick RBCs and MSB cells is located within 3 kb 5' from the α^{pi} globin gene (20). These data agree with the report of Razin *et al.* (19) who used the early labeling strand extrusion technique (41) to identify an origin at the same location.

Figure 4. α^D globin genes in CEF are replicated in the transcriptional direction. (Upper panel) Runoff replication in the absence of restriction enzyme. CEF nuclei replicated DNA *in vivo* in the presence of BrdUTP. DNA was purified, digested with *Eco*R1 and *Bam*H1 and banded on neutral CsCl. *In vivo* density labeled DNA was banded on a parallel gradient. Density labeled DNA was isolated from each gradient, electrophoresed, and blot hybridized to the α^D globin probes shown in Figure 3. (Lower panel) Runoff replication in the presence of restriction enzyme. A second aliquot of the nuclei used above was allowed to replicate DNA *in vivo* at 37°C in the presence of *Eco*R1. DNA was purified, digested with *Bam*H1 and analyzed as described in Figure 2.

Replication of the α^D globin locus in tsRSV-CEF grown at the permissive temperature

To analyze the replication of the activated α^D globin genes in RSV-CEF, nuclei were isolated from tsRSV-CEF grown at the permissive temperature (35°C) and subjected to the runoff replication assay. No exogenous replication barrier was introduced during the *in vivo* incubation, yet the normalized signal from the density labeled 1.0 kb fragment was roughly 50% less than the signal from the density labeled 0.6 kb fragment (Figure 5, top). Quantitatively similar results were obtained using nuclei from CEF transformed by wild-type RSV grown at 41°C (data removed).

The 50% reduction in the normalized signal from the 1.0 kb fragment does not convey a physical explanation for the decreased density labeling, thus it is difficult to say whether this is the maximum or minimum effect to be expected. Nevertheless, this observation is qualitatively and quantitatively reproducible in many independent experiments and, as discussed below, the

Figure 5. Alpha^D globin replication in tsNY68-CEF grown at 35°C. (Upper panel) Runoff replication in the absence of restriction enzyme. Nuclei from tsNY68-CEF [grown at 35°C] replicated DNA *in vivo*, DNA was purified, digested with *Eco*R1 and *Bam*H1 and banded on neutral CsCl. (Lower panel) Runoff replication in the presence of restriction enzyme. A second aliquot of the nuclei used above replicated DNA *in vivo* at 37°C in the presence of *Eco*R1. DNA was purified, digested with *Bam*H1, and analyzed as in Figure 4.

change in the signal ratio of 1.0 kb/0.6 kb fragments is specific in that the same assay detects no change in the *in vivo* replicative labeling of DNA fragments elsewhere in the alpha globin or the histone H5 gene loci. Hence, the observed change in runoff replication products reflects a virus-induced change in the local structure around the alpha^D globin gene.

One possible explanation for the decreased 1.0 kb fragment signal is that RSV transformation activates a cryptic origin at the 3' end of the 1.0 kb fragment. Forks would then move in the anti-transcriptional direction through the 0.6 kb and 1.0 kb fragments. If this hypothetical origin was close to the *Bam*H1 site at the end of the 1.0 kb fragment, then fewer forks might be available in the asynchronous population of nuclei for runoff labeling of the 1.0 kb fragment than for labeling of the 0.6 kb fragment.

To test whether replication forks move anti-transcriptionally from a tsRSV-induced origin downstream of the alpha^D gene, nuclei from tsRSV-CEF growing at the permissive temperature were analyzed by runoff replication in the presence of *Eco*R1.

Figure 6. Alpha^D globin replication in tsNY68-CEF grown at 41°C. (Upper panel) Runoff replication in the absence of restriction enzyme. Nuclei from tsNY68-CEF [grown at 41°C for 72 hours] replicated DNA *in vivo* at 37°C, DNA was purified, digested with *Eco*R1 and *Bam*H1 and banded on neutral CsCl. (Lower panel) Runoff replication in the presence of restriction enzyme. A second aliquot of the nuclei used above replicated DNA *in vivo* at 37°C in the presence of *Eco*R1. DNA was purified, digested with *Bam*H1, and analyzed.

Inclusion of *Eco*R1 during the runoff density labeling, instead of after DNA purification, led to a further decrease in the *in vivo* labeling of the 1.0 kb fragment relative to that of the 0.6 kb fragment (Figure 5, bottom). The depletion of the 1.0 kb fragment suggests that introduction of an exogenous barrier at the *Eco*R1 site separates the 1.0 kb fragment from the nearest origin, i.e. replication proceeds in the transcriptional direction through the 1.0 kb fragment DNA in tsRSV-CEF. Therefore, RSV infection does not induce a new replication origin 3' to the alpha^D gene.

An alternative explanation for the decrease in the 1.0 kb/0.6 kb fragment signal ratio is that RSV infection makes certain DNA hypersensitive to endogenous nuclease digestion during the *in vivo* incubation. The postulated hypersensitivity cannot be due to globin gene activity per se, since the 1.0 kb fragment signal is not diminished after runoff replication of the alpha globin genes in chick RBC nuclei (7). Nevertheless, to determine whether nuclease sensitivity secondary to RSV transformation might be responsible for the loss of the 1.0 kb fragment chromatin we analyzed unreplicated (light-light) DNA from the same CsCl gradients used in the experiments of Figures 5, 6, 8 and several control experiments. The signal ratio of these fragments in unreplicated DNA was 1.01 ± 0.06 (mean $\pm$ S.D.) when normalized to the signal ratio of these fragments density labeled

Figure 7. Histone H5 gene replication in tsNY68-CEF grown at 35°C. (Upper panel) Runoff replication in the absence of restriction enzyme. Nuclei isolated from tsNY68-CEF [grown at 35°C] replicated DNA *in vivo*, DNA was purified, digested with *HindIII* and banded on neutral CsCl. (Lower panel) Runoff replication in the presence of restriction enzyme. A second aliquot of the nuclei used above replicated DNA *in vivo* at 37°C in the presence of *HindIII*. DNA was purified, digested with *PstI*, and analyzed.

in vivo and run on parallel gradients (data removed). This indicates that the decrease in the 1.0 kb fragment signal is selective for *in vivo* replicated DNA (~1% of the total DNA in these nuclei), and therefore that RSV transformation changes the replication of the alpha globin 1.0 kb fragment chromatin.

These results also show that despite differences between the profiles of fractionated gradients in independent centrifugations, parallel gradients within a single run were very reproducible. Therefore, in RSV-CEF a 50% decrease in the normalized signal ratio of 1.0 kb/0.6 kb fragments is highly significant (greater than seven standard deviations from the mean).

RSV transformation did not activate a new replication origin 3' to the alpha globin region, nor was alpha globin chromatin degraded by nuclease *in vivo*. A third explanation for the decrease in the ratio of 1.0 kb/0.6 kb fragment signals is that alpha globin gene replication occurs in the transcriptional direction but an endogenous barrier to fork movement, near the *EcoRI* site at the end of the 1.0 kb fragment, slows replication through the 1.0 kb fragment but does not interfere with replication of the 0.6 kb fragment. A change in density labeling could arise if roughly half as many DNA polymerase molecules replicate the 1.0 kb fragment as replicate the 0.6 kb fragment during the *in vivo* incubation. Other explanations, though less likely, are also

possible. Irrespective of the molecular basis for these results, correlated with RSV transformation of CEF *in vivo* there is a change in the structure of the alpha^D globin locus which alters its runoff replication pattern.

Several additional experiments showed no change in runoff replication labeling of restriction fragments located near the origin 5' to the alpha^{pi} globin gene in nuclei from uninfected CEF or from tsRSV-CEF grown at the restrictive or permissive temperature (Y.I.-L. and M.L., unpublished); similarly, no difference in the stoichiometry of runoff replicated DNA fragments of the histone H5 gene was seen in nuclei from uninfected CEF or tsRSV-CEF (see below). Therefore, the change in replication 3' to the alpha^D globin gene is a localized phenomenon correlated with RSV infection of CEF.

Replication of the alpha^D globin locus in tsRSV-CEF grown at the nonpermissive temperature

The tsNY68 pp60^{src} protein is thermolabile at 41°C and tsRSV-CEF cultures grown at 41°C gradually lose the transformed morphology over a period of approximately 48 hours. Since RSV transformation resulted in transcription of the alpha^D globin genes and in alteration in alpha^D globin replication we tested whether pp60^{src} inactivation would restore the pattern of alpha globin replication seen in uninfected CEF.

tsRSV-CEF were cultured at 41°C for 48-72 hours, by which time the culture had regained a normal fibroblast morphology. Nuclei from these cells were submitted to the runoff replication assay at 37°C (Figure 6, top). DNA was purified, digested with *EcoRI* and *BamHI*, and analyzed as above. In contrast to the lower signal of the 1.0 kb *EcoRI*-*BamHI* fragment after replication in nuclei from cells grown at 35°C (cf. Figure 5), in nuclei from tsRSV-CEF grown at 41°C the ratio of density labeled 0.6 kb/1.0 kb fragment signals was virtually the same as that of nuclei from uninfected CEF. When *EcoRI* was added to the replication reaction to introduce an exogenous barrier between the 0.6 kb and 1.0 kb fragment DNAs, the 1.0 kb fragment signal was diminished in the density labeled DNA fraction (Figure 6, bottom), mirroring the results in nuclei from uninfected CEF. This implies that forks move in the transcriptional direction in the 3' region of the alpha^D globin locus in tsRSV-CEF grown at the restrictive temperature, as in tsRSV-CEF grown at the permissive temperature.

The differences between the runoff replication patterns using nuclei from tsRSV-CEF grown at 35°C vs. 41°C were due to the activity of the thermolabile src protein and not to the temperature shift per se, since alpha globin replication patterns were closely similar using nuclei from tsRSV-CEF grown at 35°C (Figure 5) or from wtRSV-CEF grown at 41°C (data removed). Furthermore, forks moved in the transcriptional direction through this region in nuclei from uninfected CEF grown at 35°C (Figure 4) or 41°C (data removed).

Replication of the histone H5 genes in CEF and RSV-CEF

To determine whether the changes in globin replication which accompany RSV transformation reflect a general effect on replication in RSV-CEF the replication of the histone H5 genes (Figure 3) was examined. The transcribed histone H5 genes of embryonic chick RBCs are replicated in the transcriptional direction, while the inactive H5 genes in MSB cells are replicated anti-transcriptionally (8). The present data are consistent with previous results suggesting that the inactive H5 genes are replicated anti-transcriptionally. No endogenous barrier or

selective depletion of replicated restriction fragments was detected close to the H5 genes in nuclei from tsRSV-CEF (grown at 35°C) (Figure 7, top) or uninfected CEF (data removed). When *Hind*III was added to the runoff replication mixtures reproducible depletion of the signal from the downstream 3.2 kb fragment relative to the signal from the upstream 1.3 kb fragment was seen using nuclei from tsRSV-CEF (Figure 7, bottom) or from uninfected CEF (data removed). Thus, in both uninfected and tsRSV-transformed CEF, the histone H5 genes are inactive and are replicated anti-transcriptionally.

DISCUSSION

Based on the arrangement and chronology of expression of the human globin genes, and on observations of nonrandomness during chromatin replication (42, 43), it was proposed that replication and transcription in eucaryotic chromosomes are coordinated such that active genes are replicated in the transcriptional direction. The replication polarity of inactive genes could depend on the location of the nearest transcribed gene (44). We used the induction of alpha globin gene expression by RSV to study the replication of the alpha globin locus in different transcriptional states. Our experiments extend the observation that globin mRNA is found in RSV-CEF (25) in suggesting that the induction of alpha globin expression occurs at the level of new transcription. Several other groups have used RSV infection to identify a variety of genes whose expression is induced by pp60^{src} activity (46–51); the current study is unique in demonstrating that the replication of a chromosomal DNA sequence can be altered as a corollary to the induction or repression of gene activity.

For the limited number of systems studied, virtually all copies of a given DNA sequence appear to be replicated in a preferred direction in a specific cell type. In the avian alpha globin and histone H5 genes (7, 8), and the human c-myc genes (9, 10) our data are consistent with the proposal that active genes are replicated in the transcriptional direction. Nevertheless, the replication pattern of the hamster DHFR and 2BE2121 genes (16) and possibly of other genes (e.g. see 45) may not fit this model. The available data might, however, be accommodated by a broader model in which factors which can influence replication act at DNA sites that display chromatin structural features in common with transcriptional promoter or enhancer elements.

In the present work alpha^D globin gene transcription was induced in RSV-CEF. In the same cultures a change was detected in the stoichiometry of *in vivo* replicated restriction fragments located between the alpha^D and alpha^A globin genes. A possible explanation for this result is that RSV infection changes the direction of replication from co-transcriptional in CEF to anti-transcriptional. However, a barrier inserted between the 0.6 kb and 1.0 kb fragment DNAs by *Eco*R1 addition resulted in the depletion of the 1.0 kb fragment signal during runoff replication of RSV-CEF nuclei. This suggests that the 1.0 kb fragment DNA is on the opposite side of the *Eco*R1 barrier as the nearest replication origin, i.e. that replication moves in the transcriptional direction through the alpha^D globin region in CEF and RSV-CEF. Therefore RSV infection does not activate a new origin 3' to the alpha globin locus.

RSV infection might induce a nuclease sensitive state in the 1.0 kb *Eco*R1-*Bam*H1 fragment of alpha globin chromatin. Five DNase I hypersensitive sites have been located within the alpha globin locus in chicken RBCs (22, 23). Three of these sites are

associated with the 5' promoters of the alpha^{pi}, alpha^D and alpha^A globin genes, and a fourth with the alpha globin enhancer 3' to the alpha^A gene. The 1.0 kb *Eco*R1-*Bam*H1 restriction fragment contains a fifth region of DNase hypersensitivity not associated with a known promoter or enhancer element, but located within 2.5 kb of the downstream alpha globin enhancer (23).

The data do not support the interpretation that the nuclease sensitivity of active alpha globin genes is responsible for the decrease of the 1.0 kb fragment signal in the present experiments. In chick RBCs the transcribed alpha globin genes show general DNase I sensitivity, and the 1.0 kb restriction fragment contains a DNase I hypersensitive site, yet there is no depletion of the 1.0 kb fragment during runoff replication in RBC nuclei (7). Thus, transcriptional activity, DNase (hyper)sensitivity, or runoff replication per se are not sufficient for the depletion of the 1.0 kb alpha globin fragment. Moreover, in the present experiments, the 1.0 kb fragment was not depleted in the unreplicated DNA of RSV-CEF nuclei. This observation argues that depletion of alpha globin sequences in replicated DNA is due specifically to an effect on alpha globin replication in RSV-CEF. However, it remains formally possible that the decrease in the ratio of 1.0 kb/0.6 kb fragment signals reflects a nuclease hypersensitivity in that subpopulation of alpha globin chromatin replicated during the runoff reaction, although this seems unlikely in view of the foregoing discussion.

An explanation more consistent with the available data is that during replication in RSV-CEF nuclei decreased labeling of the downstream 1.0 kb fragment occurs because RSV transformation decreases the number of DNA polymerase molecules which progress from the 0.6 kb fragment into the 1.0 kb fragment DNA. In this scenario, replication moves in the transcriptional direction through the alpha^A globin region in RSV-CEF as in CEF, but RSV infection establishes a kinetic barrier to DNA polymerase movement between the 0.6 kb and 1.0 kb fragment DNAs. This barrier is accentuated *in vivo*, and is detected as decreased polymerase labeling of the downstream 1.0 kb fragment relative to the labeling of the upstream 0.6 kb fragment. The postulated barrier to fork movement is not due to a global or irreversible distortion of replication since the effect is lost with inactivation of the pp60^{src} protein, and since no difference was detected in the replication of the histone H5 locus in nuclei from CEF or RSV-CEF. Moreover, no other alterations in replication were detected in the DNA separating the alpha^{pi} and alpha^D genes.

We favor the interpretation that the action of pp60^{src} indirectly changes the chromatin structure and slows the replication through a specific zone in the alpha globin locus, although we cannot entirely eliminate the possibility of selective nuclease sensitivity of the *in vivo* replicated chromatin. In tsRSV-CEF cultures the putative replication barrier was observed using nuclei from cells grown at 35°C but not using nuclei from cells grown at 41°C. Thus, since both runoff replication assays were performed under identical conditions at 37°C, these results show that the difference in runoff replication patterns is a reflection of a difference in globin chromatin structure established *in vivo*.

The cytoplasmic and perinuclear membrane localization of the pp60^{src} protein (52) imply that pp60^{src}-activated trans-acting factors can alter alpha globin gene expression and replication in RSV-CEF. This idea is supported by the observation that there are on average only 3–4 extra copies of the viral genome per tsRSV-CEF cell, yet under similar conditions of RSV infection >500 genes are activated (25, 31, 53). Hybridization analysis

failed to detect integration of RSV DNA within 10–15 kb of the α^D locus which might directly alter alpha globin replication. It appears therefore that viral pp60^{src} activity indirectly leads to both the induction of alpha globin transcription and the change in alpha globin replication. The decay of this pp60^{src}-induced determinant could then decrease α^D globin transcription and restore the normal α^D globin replication pattern in tsRSV-CEF grown at 41°C.

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