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# Use of recombinant DNA technology to program eukaryotic cells to synthesize rat proinsulin: A rapid expression assay for cloned genes

(rat preproinsulin/eukaryotic DNA expression vector)

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**ABSTRACT** **To use recombinant DNA technology to functionally analyze mutations introduced into cloned eukaryotic genes, a rapid procedure is necessary to assay the steps along the gene expression pathway. Since cloned rat insulin genes are not transcribed efficiently after transfection into various cell lines, I have asked whether one could drive expression by placing the insulin gene inside a transcriptional unit that functions in all mammalian cells. By using a small simian virus 40 (SV40) fragment that contains initiation signals for replication and transcription, I connected the 5′-noncoding region of the SV40 tumor antigen gene to the 5′-noncoding region of the rat insulin II gene to create a pBR322-based recombinant. If one assays shortly after its introduction into mammalian cells, it can be shown that this recombinant plasmid programs the synthesis of correctly spliced and polyadenylylated insulin mRNA that functions in the synthesis and secretion of rat proinsulin. This system permits rapid analysis of cloned *in vitro*-engineered mutations and the programming of eukaryotic cells to manufacture proteins that they normally do not synthesize.**

After a gene coding for a eukaryotic protein has been cloned and its sequence has been determined, how does one begin to correlate gene structure with control of gene expression? By using recombinant DNA technology, it is relatively simple to introduce mutations in a cloned gene; the problem is to assay the steps (transcription, RNA processing, translation, protein processing) leading to the final gene product. Recombinant DNA technology is being used to study the gene coding for the polypeptide hormone insulin (1–3). To establish the required assay, I have inserted rat insulin genes into cultured cells in a fashion that results in rat proinsulin production. This assay is rapid, thus permitting the biochemical analysis of *in vitro*-engineered mutations. This technology (programming cells to manufacture proteins that they normally do not synthesize) also may be useful in gene therapy and industry.

The two nonallelic rat preproinsulin genes have been isolated from a rat chromosomal DNA library and characterized (2). These cloned genes do not function when introduced into mouse L cells (C. Lo, personal communication). On the other hand, Gruss and Khoury (4) have obtained insulin expression by placing the complete rat preproinsulin I gene in the late region of a simian virus 40 (SV40) vector. This recombinant, together with a helper virus, will productively infect monkey kidney cells, leading to the synthesis (and secretion) of rat proinsulin; the preproinsulin mRNA in these cells is correctly spliced and possesses a 5′ terminus identical to authentic rat preproinsulin I mRNA, suggesting that RNA polymerase II is recognizing and initiating transcription of the insulin gene independent of SV40 regulatory signals. This work, in principle, provides the assay required to analyze mutations in the insulin gene; however, SV40 as a vector imposes a set of inflexible constraints (recombinant DNA size is limited by virion packaging requirements, need to use a helper virus, lytic infection in monkey cells, inability to engineer in *Escherichia coli* and transfer directly into mammalian cells) that restricts its utility.

By analogy to the methods used to obtain eukaryotic protein synthesis in bacteria (1), could one drive expression by placing the insulin gene in a transcriptional unit that functions in all mammalian cells? To address this question, I have constructed a SV40–insulin–pBR322 recombinant in which the 5′-noncoding region of the SV40 early gene [which functions constitutively (5) in most mammalian cells] is connected to the 5′-noncoding region of the rat insulin II gene.

## METHODS

**Plasmid DNA.** Genetic engineering was carried out by using pBR322 (6) and *E. coli* K-12 strains RR1 or HB101 (7). Restriction endonucleases and other enzymes were from New England Biolabs. Plasmid DNA used to transfect mammalian cells was prepared from detergent-lysed bacteria (8) followed by CsCl/ethidium bromide centrifugation and ethanol precipitation of the covalently closed circular (ccc) form. The different plasmids are described in Fig. 1.

**Transfection.** Calcium phosphate DNA precipitate suspensions were formed (9) by using plasmid DNA at 10–25 μg/ml and presented to cells by two methods. In method 1 (9), 1 ml of precipitate was added to 10 ml of fresh medium covering a nearly confluent monolayer of cells in a 10-cm dish. In method 2 (10), confluent cells from a 10-cm dish were trypsinized, centrifuged, and incubated with 1 ml of precipitate for 15 min; 10 ml of medium was added and the suspension was transferred to a 15-cm dish. In both methods, after 5 hr of incubation at 37°C in 5% $CO_2$/95% air, the DNA/medium was removed and the cells were shocked with 25% glycerol in medium for 1 min (10), rinsed, and returned to the incubator with fresh medium.

Cos cells (ref. 11; from Y. Gluzman, Cold Spring Harbor Laboratory) and HeLa cells were grown in Dulbecco's modified Eagle's medium/10% fetal calf serum. L cells and CV-1P cells were grown in Dulbecco's modified Eagle's medium/10% calf serum. All cells were incubated at 37°C in 5% $CO_2$/95% air.

**RNA.** Saline-washed cells were extracted with NaDod$SO_4$/phenol/chloroform (12) and total cellular RNA was isolated free of DNA by pelleting through CsCl (13). RNA was examined by nuclease S1 mapping (14) using end-labeled probes (15) and by blot hybridization (16).

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Abbreviations: SV40, simian virus 40; ccc, covalently closed circular (DNA); kb, kilobase(s); bp, base pair(s); T antigen, large tumor antigen.

**Protein.** Transfected Cos cells were labeled with [$^{35}$S]cysteine (4) for 4 hr and solubilized with detergent (17). The solubilized proteins were treated with antiserum to bovine insulin (Miles) in the presence and absence of excess of unlabeled bovine insulin as competitor (12). The antigen–antibody complexes were recovered (18) by using *Staphylococcus aureus* (Pansorbin; Calbiochem), extracted with 8 M urea/1% $NaDodSO_4$/0.1 M dithiothreitol at 100°C and electrophoresed on 15% acrylamide/$NaDodSO_4$ gels (19). The acrylamide gel was impregnated with fluor (EN$^3$HANCE, New England Nuclear), dried, and exposed to x-ray film at −80°C.

Insulin antigen released into the culture medium from transfected cells was measured by radioimmunoassay (Amersham). This assay uses human insulin as a standard and expresses concentrations in microunits/ml; pure human insulin is ≈25 units/mg.

## RESULTS

The rat insulin II gene contains two introns and is carried on a 3-kilobase (kb) *Pst* fragment, which has been subcloned into pBR322 (prI2; Fig. 1*a*). I have engineered a *Hin*dIII site in the 5′-noncoding region of this cloned gene and, in the process, have deleted all upstream sequences (prI2-35; Fig. 1*b*). Roberts *et al.* (20) have cloned the entire early region of SV40 (the *Hpa*II/*Bam* fragment) in pBR322 in a fashion that converted the *Hpa* II site to an *Eco*RI site (pTR405; Fig. 1*c*). I have isolated the *Eco*RI/*Hin*dIII fragment from pTR405 [the *Hin*dIII site, at nucleotide 5,175 on the SV40 genome, lies in the 5′-noncoding region of the tumor antigen gene, 12 base pairs (bp) upstream from the AUG initiation codon; this fragment also contains a functional origin of replication and both 72-bp repeats] and ligated this to *Hin*dIII-restricted prI2-35 to create the desired recombinant (prI2–SV40-46; Fig. 1*d*).

**Insulin mRNA Synthesis in Cultured Mammalian Cells.** Insulin gene expression was evaluated after introduction of the cloned genes into cultured cells by using calcium phosphate transfection. At various times after transfection, RNA was isolated and used in a nuclease S1 assay that is designed to detect correctly spliced rat insulin mRNA; in addition, a sensitive radioimmunoassay was used to measure insulin antigen released into the spent culture medium. In monkey kidney cells (CV-1P cells), both the intact insulin gene (prI2) and the deleted insulin gene (prI2-35) fail to support insulin mRNA expression, whether presented as ccc plasmids or excised (with *Pst* I) and presented as linear molecules (Fig. 2*a*, lanes 1, 2, and 5). In contrast, when prI2–SV40-46 is transfected into CV-1P cells, correctly spliced rat insulin mRNA is easily detected and insulin antigen is released into the culture medium (see below); the ccc plasmid programs this expression more efficiently than the excised (using *Hin*cII, which cuts pBR322 sequences on both sides of the SV40–insulin region) gene (Fig. 2*a*, lanes 7 and 8).

SV40 replication in monkey cells is dependent on the presence of functional large tumor (T) antigen (5). To examine the influence of SV40 T antigen on insulin gene expression, the *Eco*RI/*Bam*HI fragment from pTR405, which by itself encodes functional early region gene products (20), was introduced by cotransfection along with the different insulin gene plasmids. This procedure dramatically enhanced the level of correctly spliced insulin mRNA programmed by excised prI2–SV40-46 but only slightly stimulated expression from the ccc plasmid (Fig. 2*a*, lanes 9 and 10). These results are consistent with the idea that T antigen, supplied in *trans* by the early region gene, promotes replication of the insulin gene physically linked to the SV40 origin of replication; the resultant gene amplification leads to increased levels of insulin mRNA. Since plasmid pBR322 contains a sequence (the "poison" sequence) that inhibits rep-

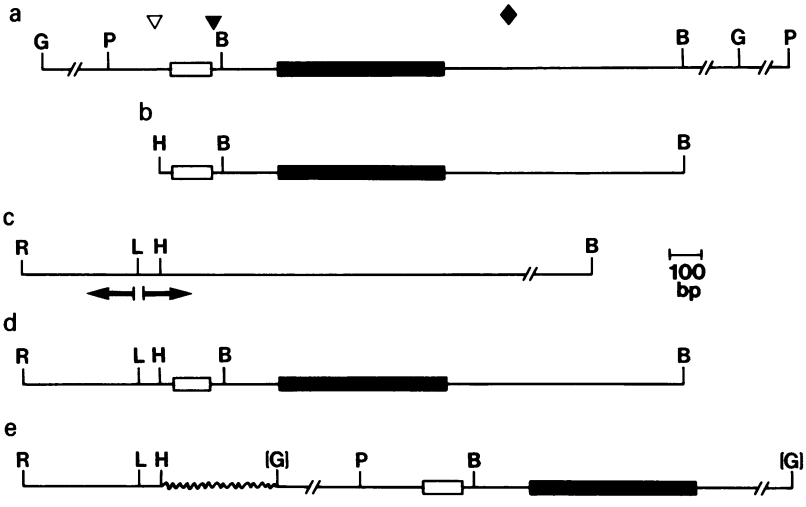


FIG. 1. Plasmids used for transfection. (*a*) prI2 and pRC11. The parent phage clone (λ Charon 4A-rI2) that carries the entire rat preproinsulin II gene (2) was used to prepare two plasmid subclones. prI2 carries a 3-kb *Pst* fragment inserted into the *Pst* site of pBR322. pRC11 contains a 4-kb *Bgl* II fragment inserted into the *Bam* site of pBR322 (constructed and supplied by R. Cate, Harvard University). ▽, 5′ End of the mature mRNA; ▼, start of the coding region; ◆, 3′ end of the mature mRNA; □, the 119-bp intron in the 5′-noncoding region; ■, the 499-bp intron in the coding region. G, *Bgl* II; P, *Pst*; B, *Bam*. (*b*) prI2-35. prI2 was used to construct a gene with the entire 5′ flanking region deleted. A *Dde* I site in the 5′-noncoding region was filled out by using the Klenow fragment of *E. coli* DNA polymerase I and ligated to *Hin*dIII linkers (Collaborative Research). The product was digested with *Hin*dIII/*Bam*, and the gene was reconstructed by ligation and inserted between the *Hin*dIII and *Bam* sites of pBR322. H, *Hin*dIII. (*c*) pTR405. This plasmid (ref. 20; supplied by T. Roberts, Harvard University) contains a functional SV40 origin of replication and the entire SV40 early region cloned between the *Eco*RI and the *Bam* sites of pBR322. Arrows, directions of late and early gene transcription. L, *Bgl* I; R, *Eco*RI. (*d*) prI2–SV40-46. The 416-bp *Eco*RI/*Hin*dIII fragment from pTR405 was ligated to *Eco*RI/*Hin*dIII-cut prI2-35 to create prI2–SV40-46. The nucleotide sequence across the *Hin*dIII site (5′→3′, C-T-T-T-G-C-A-A-A-A-A-G-C-T-T-G-G-T-A-A-G-T-G-A-C-C-A) shows that the SV40 sequence is in the correct orientation and connected by the *Hin*dIII linkers to the filled out *Dde* I site. (*e*) prI2–SV40-15. The *Eco*RI/*Hin*dIII fragment from pTR405 was ligated to *Eco*RI/*Hin*dIII-cut pRC11 to create prI2–SV40-15 (∿, the 346 bp of pBR322 sequence that separate the SV40 origin from the beginning of the rat sequences). This construction was also carried out by using the pBR322 derivative pXF3 (constructed and supplied by D. Hanahan, Harvard University) in which the replication inhibitory poison sequence has been removed, creating the recombinant prI2–SV40-15–XF3.

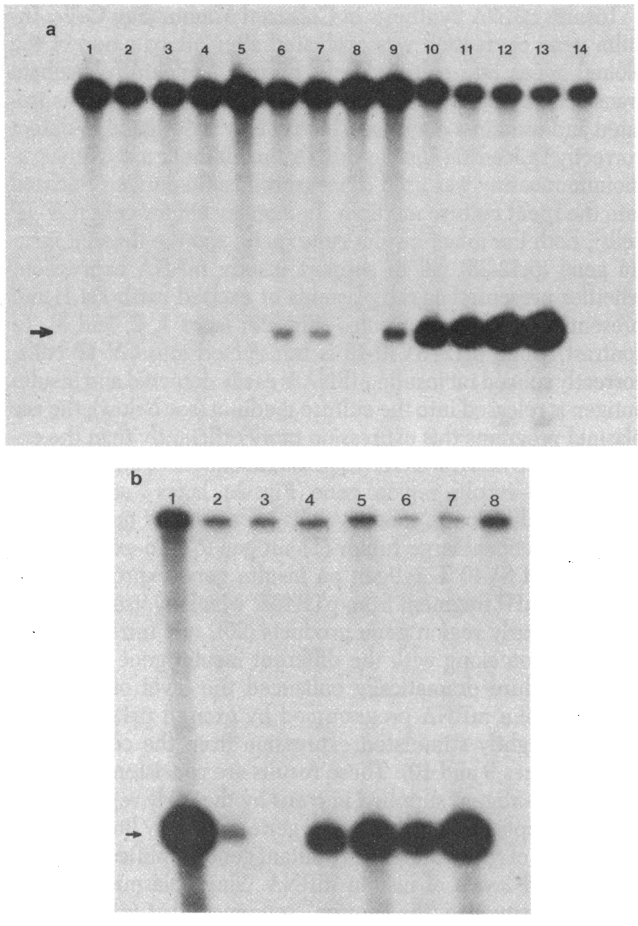


FIG. 2. Nuclease S1 assay of insulin mRNA from transfected cells. RNA from transfected cells was hybridized with an insulin-specific end-labeled probe at 49°C (14, 15). After treatment with nuclease S1 and ethanol precipitation, the digestion products were resolved on a 6% acrylamide/7 M urea gel and autoradiographed. The probe used was a 750-bp fragment from the insulin II gene uniquely 3'-end labeled at a *Bam* site in the coding region and extending to an *Hha* site on the other side of the 499-bp intron in the coding region. If a correctly spliced mRNA hybridizes to the labeled antisense DNA strand, nuclease S1 will digest the unhybridized intronic sequences, leaving a protected 170-nucleotide labeled fragment (arrows). The amount of probe used (0.04 pmol) for each RNA sample represents an excess of DNA; hence the assay quantitatively measures insulin mRNA levels. (*a*) CV-1P cells (lanes 1–10) were transfected (method 1) with plasmid DNA (12 μg of each DNA per 10-cm dish); the DNA was either ccc or digested with the denoted restriction enzyme, phenol extracted, and ethanol precipitated. RNA was isolated from the dishes after 2 days and one-half of the sample was used for the assay. Lanes: 1, prI2; 2, *Pst* I-cut prI2; 3, prI2 and *Eco*RI/*Bam*HI-cut pTR405; 4, *Pst* I-cut prI2 and *Eco*RI/*Bam*HI-cut pTR405; 5, *Pst* I-cut prI2-35; 6, *Pst* I-cut prI2-35 and *Eco*RI/*Bam*HI-cut pTR405; 7, prI2–SV40-46; 8, *Hinc*II-cut prI2–SV40-46; 9, prI2–SV40-46 and *Eco*RI/*Bam*HI-cut pTR405; 10, *Hinc*II-cut prI2–SV40-46 and *Eco*RI/*Bam*HI-cut pTR405; 11–13, rat preproinsulin mRNA standards contained in 10, 20, and 30 ng, respectively, of rat insulinoma poly(A)$^+$RNA subjected to nuclease S1 analysis; 14, control (no cellular RNA was added to the assay mixture). (*b*) Cos cells (lanes 1–4) were transfected (method 2) with plasmid DNA at 25 μg per 15-cm dish. RNA was isolated from the dishes after 3 days and one-quarter of the sample used for the assay. Lanes: 1, prI2–SV40-46; 2, prI2–SV40-15; 3, prI2; 4, prI2–SV40-15–XF3; 5, prI2–SV40-46 (from another experiment); 6, 10 ng of rat insulinoma poly(A)$^+$RNA; 7, 50 ng of rat insulinoma poly(A)$^+$RNA; 8, control (no RNA was added to the assay mixture). This autoradiogram was overexposed to show that prI2–SV40-15, but not prI2, programs the synthesis of correctly spliced insulin mRNA. Radioimmunoassay showed the presence of insulin antigen at 227 microunits/ml in the spent medium (sampled at the time of RNA isolation) from culture 1; media from cultures 2–4 were below the sensitivity of the assay (<15 microunits/ml).

lication in monkey kidney cells (21–24), the increased insulin mRNA synthesis programmed by excised prI2–SV40-46, relative to that by intact ccc plasmid, supports the copy number–gene expression relationship. Curiously, cotransfection of the *Eco*RI/*Bam*HI fragment from pTR405 with excised prI2 or with excised prI2-35 resulted in insulin mRNA appearance (Fig. 2*a*, lanes 4 and 6). Considering that recombination between transfected DNA molecules is a rather active process (25–27), it is possible, in the cotransfection experiments, that the SV40 origin becomes spliced adjacent to the insulin coding region, resulting in replication and eventual insulin mRNA synthesis (28).

To simplify the introduction of plasmids into an environment where they can replicate and to eliminate the problem of recombination between heterologous transfected DNA molecules, the insulin plasmids were examined in Cos cells (11). These cells are derivatives of CV-1 cells that constitutively synthesize T antigen and thus are capable of supporting the replication of SV40 *tsA* mutants (11) and recombinants that carry a SV40 origin of replication (23, 24, 28). When prI2–SV40-46 is introduced into Cos cells, correctly spliced insulin mRNA is easily detected (Fig. 2*b*, lanes 1 and 5); prI2 (lane 3) and prI2-35 (data not shown) are absolutely incapable of supporting this expression. Surprisingly, if the poison sequence is removed from the pBR322 portion of prI2–SV40-46, insulin mRNA does not accumulate to higher levels in Cos cells (data not shown).

Recently, Mellon *et al.* (28) showed that pBR322 recombinants carrying a SV40 origin and an intact globin gene replicate efficiently and program correctly spliced globin mRNA synthesis in Cos cells; presumably, this synthesis is the result of RNA polymerase II recognizing the globin gene promotor since the 5' end of the globin transcripts is identical to authentic globin mRNA. To examine the analogous situation, plasmid prI2–SV40-15, which contains a SV40 origin separated from the insulin II gene (Fig. 1*e*), was constructed; when introduced into Cos cells, this plasmid inefficiently programs insulin mRNA synthesis (Fig. 2*b*, lane 2). When the poison sequence is removed (Fig. 1*e*), prI2–SV40–XF3 programs insulin mRNA synthesis more efficiently (Fig. 2*b*, lane 4), albeit not as well as prI2–SV40-46 (see below and Table 1).

The results in Fig. 2 show that prI2–SV40-46 is capable of programming the synthesis of correctly spliced insulin mRNA in cells that are permissive for SV40 infection irrespective of the presence of T antigen. In cells incapable of supporting SV40 replication (e.g., HeLa and L cells), prI2–SV40-46 programs insulin mRNA synthesis, but prI2 and prI2-35 do not (data not shown). These data are in accord with the observation that the SV40 early region promotor functions constitutively in most mammalian cells (5). Since Cos cells support the highest levels of expression, insulin gene expression in this system was examined in greater detail.

**Insulin Expression in Cos Cells.** In Cos cells, the insulin mRNA synthesized from prI2–SV40-46 is polyadenylylated and slightly larger than authentic rat insulin mRNA (a mixture of preproinsulin mRNA I and mRNA II) as shown by hybridization blot analysis. The transfected cell insulin mRNA has both introns spliced exactly as found in authentic rat preproinsulin mRNA II (data not shown); however, the 5' end maps as a heterogeneous set of termini ≈76–90 nucleotides from the *Hin*dIII site in the 5'-noncoding region (Fig. 3). Early in a SV40 lytic infection, early SV40 mRNA 5' termini map heterogeneously ≈60 nucleotides upstream from the *Hin*dIII site in the 5'-noncoding region of the tumor antigen gene (29). Later in the infection, the early gene transcripts possess 5' termini that map even further upstream [≈100 nucleotides from the *Hin*dIII site (30)]. This shift of transcriptional initiation during a SV40 infection, and the 5' termini observed in Cos cells, may be ex-

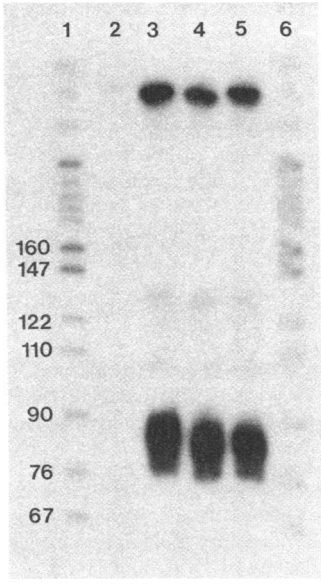


FIG. 3. Mapping the 5′ ends of RNA from transfected Cos cells. Cos cells were transfected (method 2) with prI2–SV40-46 at 20 μg of DNA per dish; RNA was isolated after three days. Assays were carried out as described in Fig. 2 using various amounts of nuclease S1 and a 5′-end-labeled probe extending upstream from the *Hin*dIII site of prI2–SV40-46. The digestion products were resolved on an 8% sequence analysis gel. Lanes: 1 and 6, $M_r$ markers ($^{32}$P-labeled *Hpa* II-cut pBR322; chain length in nucleotides shown alongside); 2, control (3 μl of nuclease S1; no RNA was added to the assay mixture); 3, transfected cell RNA (1/20th of the material from a 15-cm dish) and 3 μl of nuclease; 4 and 5, transfected cell RNA as in lane 3, 4 and 5 μl, respectively, of nuclease.

plained by a model relating the cooperative binding of T antigen to the control of early transcription (30, 31).

How much rat insulin mRNA is synthesized in the Cos cells? Nuclease S1 assays show that correctly spliced rat insulin mRNA

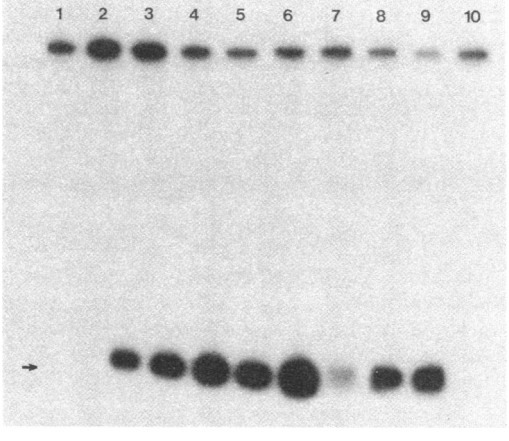


FIG. 4. Nuclease S1 assay of insulin mRNA from transfected cells: Time course of correctly spliced insulin mRNA accumulation programmed by prI2–SV40-46. Cos cells (lanes 1–6) were transfected (method 1) with prI2–SV40-46 at 15 μg of DNA per 10-cm dish. RNA was isolated from the dishes at various times and one-half of the sample was used for the assay as described in Fig. 2. Lanes: 1, 8 hr; 2, 20 hr; 3, 32 hr; 4, 44 hr; 5, 56 hr; 6, 68 hr; 7–9, 10, 20, and 30 ng, respectively, of rat insulinoma poly(A)$^+$RNA was added to the assay mixture; 10, control (no RNA was added to the assay mixture). Radioimmunoassay showed the presence of insulin antigen at 22.4, 25.4, 62.6, 77.1, 129, and 184 microunits/ml in the spent media from cultures 1–6, respectively.

Table 1. Insulin mRNA concentrations in Cos cells after plasmid transfection

| | mRNA |
|---|---|
| prI2–SV40-46 | 500–1,000 |
| prI2–SV40-15 | ≈3 |
| prI2–SV40-15–XF3 | ≈30 |

Insulin mRNA concentrations (no. of molecules per cell) were estimated by comparison of nuclease S1 assay results (Fig. 2) using rat insulinoma poly(A)$^+$RNA as a standard. The calculation assumes that a rat insulinoma cell contains 20 pg of RNA, 2% of which is poly(A)$^+$RNA and that preproinsulin II mRNA (600 nucleotides long) represents 0.15% of the poly(A)$^+$RNA (1); hence, there are $\approx 5 \times 10^7$ molecules of preproinsulin II mRNA in 10 ng of rat insulinoma poly(A)$^+$RNA.

continues to accumulate for 3 days after transfection (Fig. 4); this is in contrast to the results in CV-1P cells in the absence of T antigen, where insulin mRNA levels do not increase with time. By using rat insulinoma poly(A)$^+$RNA as a standard to quantitate the nuclease S1 assays, 3 days after transfection, one finds 500–1,000 molecules of insulin mRNA per Cos cell (Table 1); this calculation is uncorrected for the fact that only a small fraction of the cells have actually taken up functional DNA molecules (10).

When transfected Cos cells are labeled with [$^{35}$S]cysteine and the solubilized proteins are exposed to insulin antiserum, one detects, after NaDodSO$_4$/polyacrylamide gel electrophoresis and autoradiography, a single polypeptide in prI2–SV40-46-transfected cells (Fig. 5, lane 1) that is not found in prI2-35-transfected cells (lane 3). Excess unlabeled insulin prevents the binding of this polypeptide to the insulin antiserum (lane 2).

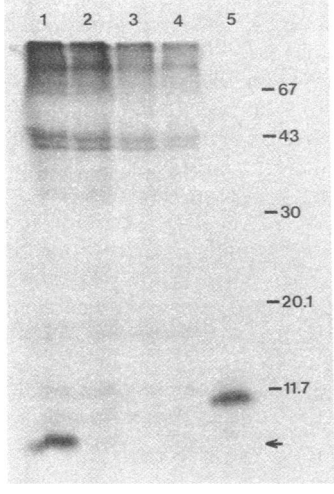


FIG. 5. Polyacrylamide gel electrophoresis of insulin-immunoprecipitable proteins from transfected Cos cells. Cos cells were transfected (method 2) with plasmid DNA and plated in 6-cm dishes. After 3 days, the cells were labeled with [$^{35}$S]cysteine at 200 μCi/ml for 4 hr and then immunoprecipitated and electrophoresed in a NaDodSO$_4$/polyacrylamide gel. Rat insulinoma poly(A)$^+$RNA was translated in a HeLa cell-free extract (cell-free translation was kindly carried out by M. Morgan and A. Shatkin, Roche Institute of Molecular Biology), and the products were similarly analyzed. Lanes: 1, prI2–SV40-46 (1/10th of the cell extract was used for this analysis); 2, as in lane 1, except that the immunoprecipitation was carried out in the presence of 10 μg of bovine insulin; 3, prI2-35 (1/10th of the cell extract was used); 4, as in lane 3, except that 10 μg of bovine insulin was also present; 5, cell-free translation products from rat insulinoma poly(A)$^+$RNA. Bovine proinsulin (arrow) and $M_r$ (shown $\times 10^{-3}$) markers were run in a parallel lane and visualized by Coomassie blue staining.

This polypeptide has an electrophoretic mobility similar to that of bovine proinsulin (8,700 daltons) and is smaller than authentic rat preproinsulin (11,500 daltons; Fig. 4, lane 5). Cell-free translation of RNA from prI2–SV40-46-transfected cells generates an insulin-immunoreactive polypeptide that comigrates with rat preproinsulin (data not shown). Insulin radioimmunoassay on spent culture media from prI2–SV40-46-transfected cells detects insulin antigen (e.g., Figs. 2*b* and 4); in a 3-day period after transfection, ≈80 pmol of insulin antigen is released from $10^7$ cells. Labeling with [$^{35}$S]cysteine shows that the protein secreted from the cells is the same size as that found inside the cell (data not shown).

Hence, in Cos cells, the hybrid SV40–rat insulin mRNA appears to be translated into rat preproinsulin II that is rapidly processed (by removal of the signal sequence) to proinsulin. The processing and secretion of rat proinsulin II programmed by prI2–SV40-46 is consistent with the observations of Gruss and Khoury (4) on rat proinsulin I production in monkey kidney cells.

## DISCUSSION

These results show that a fragment of the SV40 genome carrying a functional origin of replication and the early region promotor is capable of driving expression of the rat insulin II gene. Plasmid prI2–SV40-46 functions most efficiently in Cos cells, where replication activated by the constitutively supplied T antigen generates many copies of the recombinant genome a very short time after transfection (23, 24, 28); the resultant gene amplification and SV40-promoted transcription permits analyzing insulin gene expression immediately (12–72 hr) after transfection. Mutations can be introduced into the insulin gene portion of prI2–SV40-46, and the individual variants can be isolated after cloning in *E. coli* and rapidly assayed after transfection into Cos cells (32). This sytem has proven convenient for the rapid analysis of cloned *in vitro*-engineered mutations because the recombinant plasmids are transferred directly from bacterial to mammalian cells; in addition, unlike first-generation SV40 vector systems, there is no restriction on genome size or need to use a helper virus. One serious disadvantage in using the SV40 early region promotor to force expression is that this approach precludes studying transcription of the gene in question. The related system recently described by Mellon *et al.* (28) is not restricted in this sense and clearly is the method of choice to analyze mutant genes; however, this system works only in Cos cells and, for unexplained reasons, certain genes [β-globin (28) and rat insulin II] are poorly transcribed and translated (28).

Recently, it has been shown (33–36) that the SV40 early region promotor, in conjunction with other regulatory signals (for splicing and polyadenylylation), will drive expression of several prokaryotic genes and eukaryotic cDNA clones in both monkey (replicative mode) and nonsimian (nonreplicative mode) cells. The work described here confirms their observations that the SV40 early region promotor will function in the absence or presence of T antigen to transcribe adjacent sequences on a pBR322 plasmid vector. In addition, this work shows that no regulatory signals other than the early region promotor need be used to force expression of a cloned eukaryotic gene that possesses its own introns, transcriptional termination, and polyadenylylation signals.

Although the question has not been examined here, based on the transcriptional behavior of integrated SV40 genomes (5, 33–36), one would predict that prI2–SV40-46 would continue to program (pro)insulin biosynthesis after selection for a transformant carrying the recombinant plasmid integrated into high molecular weight DNA. In this fashion, one could stably program cells to manufacture proteins that they normally do not synthesize.

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