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Effect of Intercistronic Length on Internal Ribosome Entry Site (IRES) Efficiency in Bicistronic mRNA

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Specific structures found in the mRNA of picornavirus are known to allow a cap-independent translation. These structures, named internal ribosome entry sites (IRES), are also able to favor translation of the second cistron in bicistronic mRNAs. Their mechanism of action is not well understood. In the present study, two IRESs have been used: the IRES from poliovirus and a newly discovered IRES (SUR) composed of the 5′ P untranslated sequence from SV40 early genes, the R structure, and a small part of the U5 region from the human leukemia virus-1 (HTLV-1). The bicistronic constructs containing the firefly luciferase gene as the first cistron and the chloramphenicol acetyltransferase (CAT) as the second cistron were driven by the Rous sarcoma virus (RSV) promoter and contained the early gene SV40 terminator. All the resulting plasmids were tested by transfection in HeLa and CHO cells. In the bicistronic mRNAs without IRES, the expression of the CAT gene was dependent on the distance between the two cistrons. The maximum efficiency in the expression of the second cistron was obtained when the intercalating RNA was composed of 30 to 90 nucleotides. This expression was deeply reduced when the intercalating fragment contained 8 or 300 nucleotides and was undetectable with 500 nucleotides. Unexpectedly, the luciferase mRNA was almost not expressed when the intercalating RNA was of 8 or 30 nucleotides. Expression of the luciferase gene occurred when the intercistronic RNA fragment was of 80 nucleotides and it became lower at 300 and 500 nucleotides. The same observations were done when the poliovirus or the SUR IRESs were added after the intercistronic spacers. However, expression of the CAT gene was amplified by both IRESs. When the CAT cistron preceded by the poliovirus or SUR IRES was introduced within luciferase cistron, 316 nucleotides before its termination codon, the IRESs were able to initiate translation of the following CAT gene irrespectively of the mRNA luciferase reading frame. Moreover, with all these constructs the highest expression level of the CAT cistron did not exceed 10% of that obtained with the same vector carrying only the CAT cistron. To identify a possible relation between the IRESs and the cap site, the CAT cistron preceded or not with an IRES was introduced 210 nucleotides downstream of the AUG codon of the luciferase gene (i.e., 258 nucleotides from the cap site) and 100 nucleotides after an added UAG termination codon. Expression of the CAT gene was not modified by the addition of the poliovirus IRES but it was strongly stimulated by the SUR IRES (the level of expression corresponded to 65% of that obtained with the same vector carrying only the CAT cistron). These results suggest that there is a cooperation between the cap and the SUR IRES and not the poliovirus IRES to stimulate translation. These data indicate that IRESs must be introduced in precise position to allow an efficient expression of the second cistron in bicistronic mRNAs.

Internal ribosome entry site (IRES) Translation Bicistronic mRNA

IN most eucaryote mRNAs, the 40s ribosomal subunit binds to the cap structure with initiation factors and moves along to a AUG codon surrounded by a consensus sequence, according to a scanning mechanism (13). The scanning efficiency is greatly reduced by the presence of secondary structure in the 5′ un-

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translated region (5′ UTR) of mRNA (14). A certain number of mRNAs do not follow this rule and they are quite efficiently translated despite the presence of highly structured region in their 5′ UTR. This is the case for the mRNA from picornaviruses that, in addition, have the capacity of initiating translation in a cap-independent manner (12). Instead of the cap, these viruses use a viral protein covalently bound to the 5′ P end of some of their mRNAs. This allows these mRNAs to be translated efficiently even when the cap of the cellular mRNA is inactivated by the infection. This is particularly the case for the poliovirus, which expresses a protease, the proteinase 2A, inactivating the initiation factor eIF4G (23). The fact that the picornavirus 5′ UTR was able to work independently of the cap suggested that it might initiate de novo translation of second cistrons in biscistronic mRNAs. These conclusions rely on several complementary experimental data. In cell-free system, picornavirus 5′ UTR can trigger second cistron translation in bicistronic mRNA devoid of cap (12). A hairpin loop introduced before the first cistron, which reduces its translation, does not prevent translation of the second (27). A cistron introduced after the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) in a circular RNA can be translated in a cell-free system (5).

RNA sequences having IRES property have been found later in other viruses such as murine hepatitis virus (29), hepatitis C virus (25), and pestivirus (24). Although these IRESs do not have clear similar structures, they have common properties. The hepatitis C IRES can thus efficiently replace poliovirus IRES (17). Recently, IRESs have also been found in two retroviruses: the Moloney (30) and the Friend (4) leukemia viruses. We also found that a classical IRES activity was located in a HTLV-1 RNA fragment containing the R region and the following 39 nucleotides of the U5 region (1). The IRES property of this HTLV-1 fragment was established by the fact that it strongly favored the expression of the second cistron in bicistronic mRNAs and that it could act despite the presence of an RNA loop before the first cistron (1). The stimulatory effect of the HTLV-1 fragment was also shown to be amplified by the presence of the SV40 early gene untranslated region, forming a quite efficient IRES named SUR (1). Several years ago, the HTLV-1 RNA fragment was shown to stimulate translation of monocistronic mRNAs when added after the early gene SV40 promoter (28). We also observed that the SV40 5′ UTR, associated with the HTLV-1 R region, enhanced expression with all the tested promoters in various but not all cell types (2).

As could be expected, several cellular mRNAs having an IRES have been found. This suggested that the viruses use a normal cellular mechanism that allows translation of some mRNAs in physiological situations when the cap has become nonfunctional. This is the case of the immunoglobulin heavy chain binding protein (BiP) (26), the Antennapedia (22), the β-kinesin (6), and the human fibroblast growth factor 2 mRNA (20).

The use of IRES is helpful to generate functional bicistronic mRNA to be translated in transfected cells or in transgenic animals (20). The conditions to generate optimized vectors are not known. In preliminary experiments we noticed that both cistrons separated by an IRES did not work independently in all cases. The distance between the termination of the first cistron codon, the IRES, and the initiation codon of the second cistron appeared important. This parameter in bicistronic mRNA seems essential, although the rules have not been completely defined (19,20). The present work has been undertaken to tentatively determine the interference of the distance between the cistrons separated by an IRES in bicistronic mRNAs. For this purpose, the classical poliovirus and the recently identified HTLV-1 (SUR) IRESs have been used.

MATERIALS AND METHODS

Plasmid Constructs

All the plasmids contained the Rous sarcoma virus (RSV) (10) promoter and the early gene SV40 (Simian virus) terminator. The firefly luciferase (7) and the chloramphenicol acetyltransferase (CAT) genes (21) were used as reporter genes.

The plasmid pRSVLuc contained only the luciferase gene. The plasmids constructed for the present study contained the firefly luciferase gene as the first cistron and CAT as the second cistron.

The plasmids p247 contained a 7-nucleotide spacer between the luciferase gene termination codon and the initiation codon of the CAT gene.

The plasmid p290 was obtained by replacing the *Xba*I-*Asp* 718 fragment from the plasmid p247 by the *Sal*I-*Sac*I fragment of the polylinker from the plasmid pGEM-luciferase (Promega). The *Asp* 718 and *Sac*I sites were rendered blunt before the cloning.

The plasmid p243 contained the sequence following the luciferase gene in the plasmid pGEM luciferase up to the blunt *Sac*I site.

The *Pvu*II-*Pvu*II fragment of the plasmid p-Bluescript was dimerized. The 430-bp *Xho*I-*Xho*I fragment isolated from the *Pvu*II dimer was inserted into the *Xho*I site in the spacer of the plasmid p243. The resulting construct was the plasmid 236′.

The plasmid p239′ derived from the p236′ after withdrawal of the *Bss*HII fragment.

The plasmids p249, p292, p244, p237′, and p231′ were similar to p247, p290, p243, p239′, and p236′ with the poliovirus IRES intercalated after the spacers.

The plasmids p248, p291, p245, p258′, and p234′ were also similar to p247, p290, p243, p239′, and p236′ with the SUR IRES intercalated after the spacers. The only difference is that the DNA fragment from the pGEM luciferase polylinker was taken up to the blunt site *Sfi*I instead of *Sac*I.

The plasmids p270, p271, and p272 contained the *Xho*I-*Xho*I fragment from the plasmid pR harboring a fragment of 196 nucleotides following the termination codon of the whey acidic protein (WAP) gene. This sequence obtained by PCR was cloned in the *Sma*I site of the plasmid pPoly III. It was introduced in reverse orientation in the plasmids p270, p271, and p272. The resulting spacer contained 293 nucleotides.

All these constructs are shown in Fig. 1. The spacers in the different constructs contained 7 or 9, 29 or 30, 86 or 87, 343 or 344, and 516 or 517 nucleotides. None of these spacers contained a termination codon, which might interrupt translation.

The plasmid p273 contained the CAT gene introduced into the *Xba*I site of the luciferase gene at 110 bp downstream of the initiation codon of the luciferase gene (i.e., 158 nucleotides from the cap site and just after an added UAG termination codon). The plasmids 273′ and 274 were similar to p273 but they contained the poliovirus and the SUR IRES, respectively, added before the CAT gene. The plasmids 273, 273′, and 274 derived from p239′, p237′, and p238′, respectively (Fig. 1), after withdrawal of their *Xba*I-*Xba*I fragments and religation. These constructs are shown in Fig. 4A.

The plasmids p251, p251′, and p250′ derived from the plasmids p239′, p237′, and p238′, respectively. They were obtained by removing the *Eco*RV-*Eco*RV fragment from the luciferase gene and religating the resulting open plasmids.

Similarly, the plasmids p253, p253′, and p252′ derived from the plasmids p239′, p237′, and p238′, respectively. They were obtained by removing the *Eco*RV-*Spe*I fragments and religating the resulting plasmid after blunting the *Spe*I site (the *Eco*RV site was in the luciferase gene and the *Spe*I site in the spacers).

The plasmids p255, p255′, and p254′ derived from the plasmids p239′, p237′, and p238′, respectively. They were obtained by removing the *Eco*RV-*Not*I fragment and religating the resulting plasmid after blunting the *Not*I site (the *Eco*RV site was in the luciferase gene and the *Not*I site in the spacers).

In this way, in the three groups of plasmids, the CAT gene had the three different possible reading frames of the luciferase cistron. These constructs are shown in Fig. 5A.

Cell Transfection and Determination of the Enzymatic Activities

HeLa and CHO cells were cultured at 50% confluency and transfected using lipofectamine (BRL) as recommended by the manufacturer. Three dishes (60 mm diameter) were transfected independently with each plasmid.

The luciferase and CAT activities were measured in cell extracts 2 days after transfection according to methods previously described (7,21). Each determination was done in duplicate. Results are the means ± SEM of the data obtained from three independent dishes. They are expressed as units per dish.

Northern Blot Analysis

Transfections were carried out in CHO as depicted above. Two days later, total RNA was extracted using guanidinium isothiocyanate, chloroform, and phenol at acid pH or with the kit provided by Boehringer Mannheinn. RNA was submitted to agarose gel electrophoresis and transferred to nylon membranes. The mRNA for CAT was revealed using a ^{32}P-labeled probe (30.106 cpm/ng DNA) at the concentration of 3×10^6 cpm/ml in a buffer containing 0.5 M sodium phosphate, 7% sodium dodecyl sulfate. These techniques have been described in a previous article (1). The content of total RNA in each lane was checked by illumination of the nylon membrane with UV light after the transfer and before hybridization.

RESULTS

Spacer Length Modulates Expression of the Cistrons

Plasmids harboring the luciferase and the CAT genes separated by spacers containing 7 or 9, 29 or 30, 86 or 87, 343 or 344, or 516 or 517 nucleotides were constructed. Some constructs had no IRES sequence after the spacer, the others contained at this site either the poliovirus IRES or the HTLV-1 IRES (Fig. 1). These constructs were transfected into HeLa and CHO cells.

In the absence of IRES both cistrons were poorly expressed or not expressed with a 7-nucleotide spacer in HeLa cells. They were maximally expressed with a spacer of 86 nucleotides (Fig. 2A), a result compatible with the data obtained by Kozak (15), who observed that the second cistron of bicistronic mRNAs was maximally translated with a similar spacer

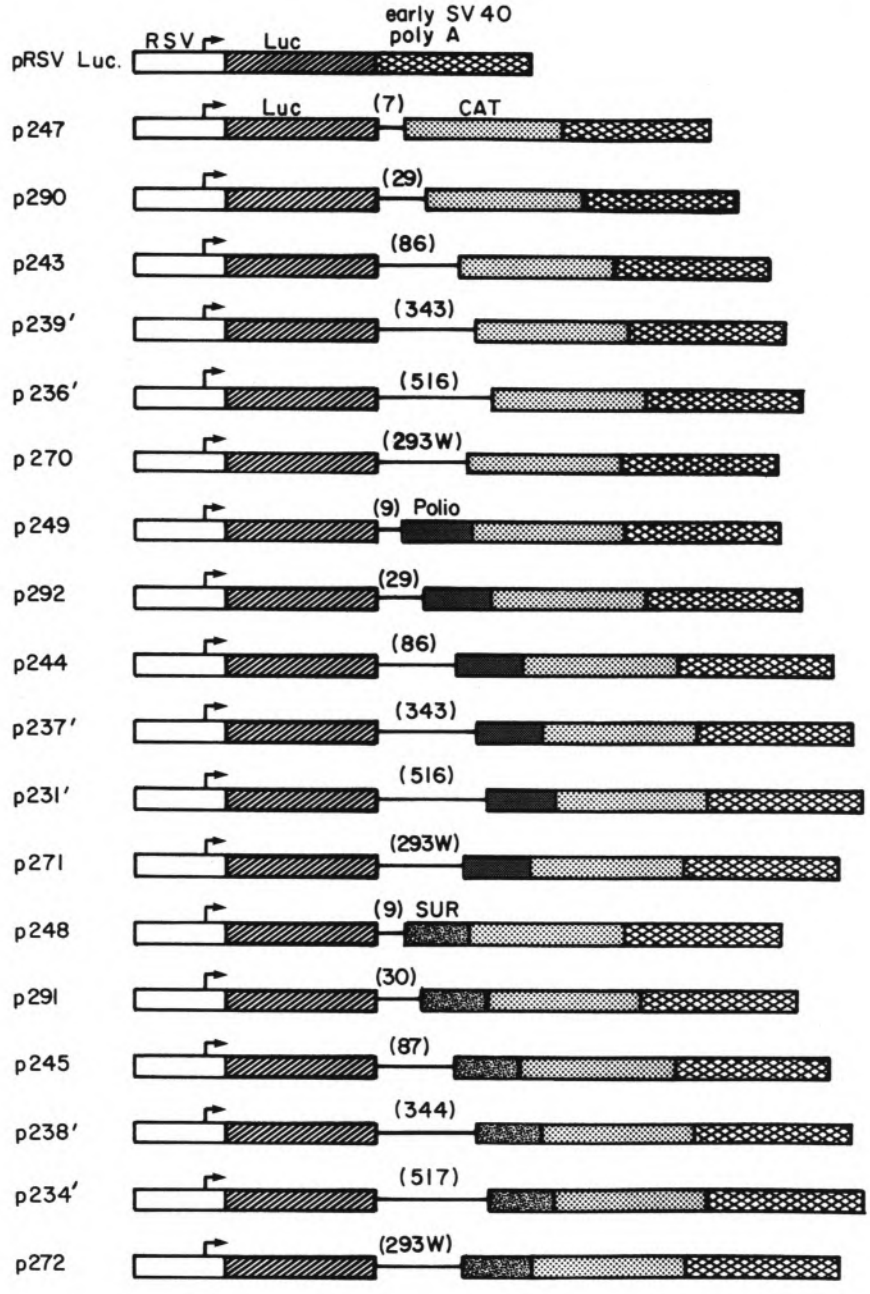

FIG. 1. Schematic representation of the bicistronic constructs with spacers of different length. Luc and CAT designate the firefly luciferase and chloramphenicol acetyltransferase genes, respectively. RSV is the LTR from Rous sarcoma virus. The arrow indicates the beginning of transcription. The numbers between the cistrons indicate the nucleotide numbers of the different spacers. All the spacers were fragments of the pGEM luciferase and pBluescript plasmids except for the 293 W, which is a fragment of the rabbit WAP gene (see Materials and Methods). The length of the different fragments of the constructs is not on scale. The length is designated by the numbers in parentheses.

length. With longer spacers (343 and 516 nucleotides) the expression of both cistrons strongly decreased. In the presence of both IRESs, the pattern of expression was essentially the same with a maximum expression when the spacer was of 86 or 87 nucleotides. Unexpectedly, the second cistron, but also the first, were less and less expressed when the spacers were longer (Fig. 2B, C).

The same gene constructs plus those containing 29 or 30 nucleotides spacers were tested in CHO cells. Results shown in Fig. 3 are compatible with those obtained with HeLa cells. The maximum expression of the first cistron was obtained with the 86–87-nucleotide spacers irrespective of the presence of the IRES. With a spacer of 29 or 30 nucleotides and in the absence of IRES, the second cistron was maxi-

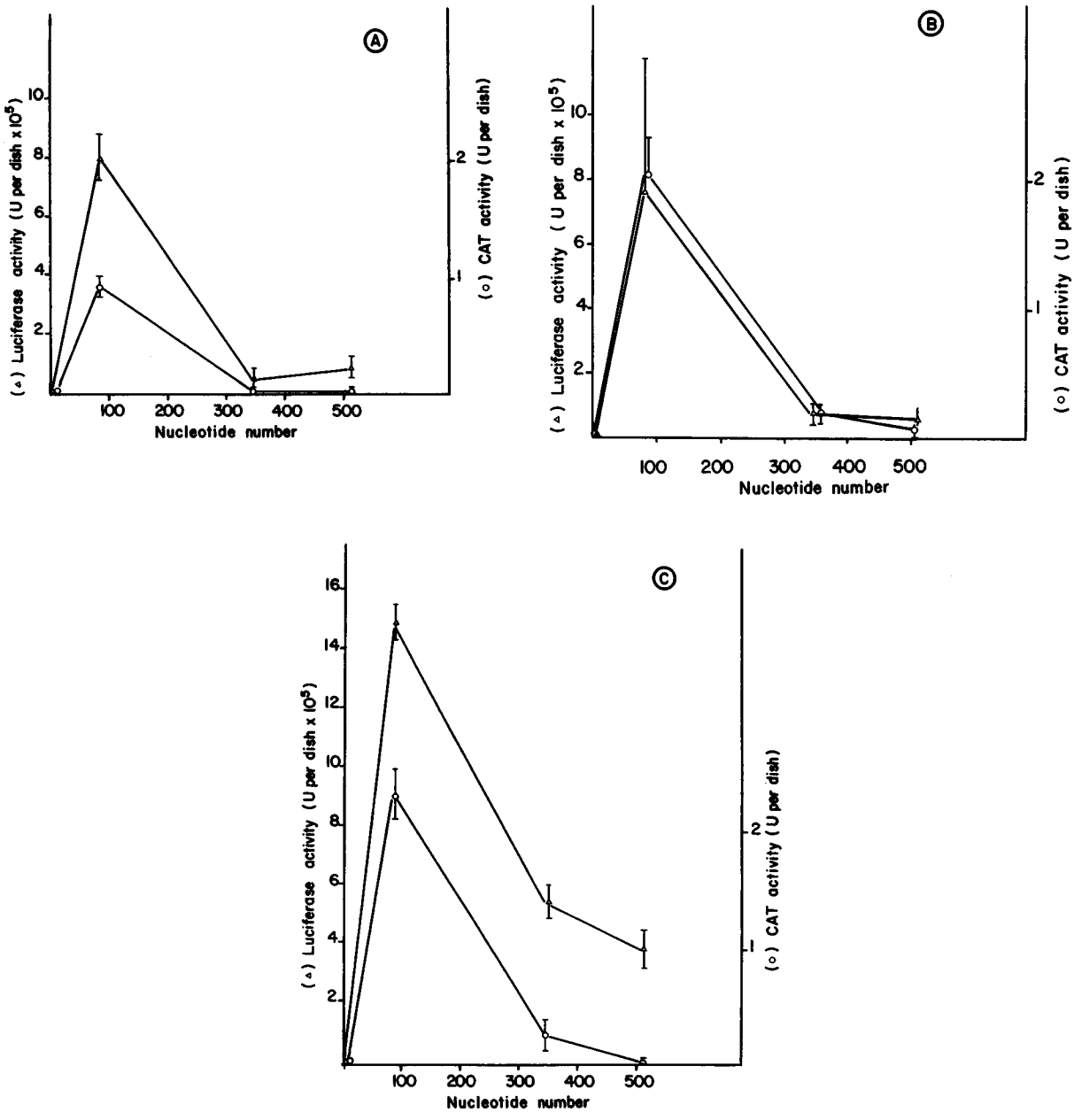

FIG. 2. Effect of the spacer length on the expression of the luciferase and CAT cistrons in HeLa cells. The abscissa indicates the nucleotide numbers of the spacers. The enzymatic activities are the means ± SEM from three independent dishes. (A) Spacers alone, (B) spacers followed by the poliovirus IRES, and (C) spacers followed by the SUR IRES.

mally expressed, confirming Kozak's results (15). The highest expression of the second cistron added after the IRES was obtained with the 29–30-nucleotide spacers. Results not shown here confirmed these results when another IRES (from the encephalomyocardiditis virus IRES) was added between the two cistrons.

In all cases, the first cistron was poorly expressed with the 29–30-nucleotide spacers. As in HeLa cells, the expression of the two genes was progressively reduced as the spacers became longer than 86–89 nucleotides. To check if this reduction was not due to some unspecific and unknown signals contained in the spacers, another sequence, a 293-nucleotide fragment from the rabbit WAP gene terminator, was intercaled between both cistrons. The same reduction of expression was obtained, with and without IRES.

Data not shown here indicated that the same observations were obtained when the EMCV IRES was used.

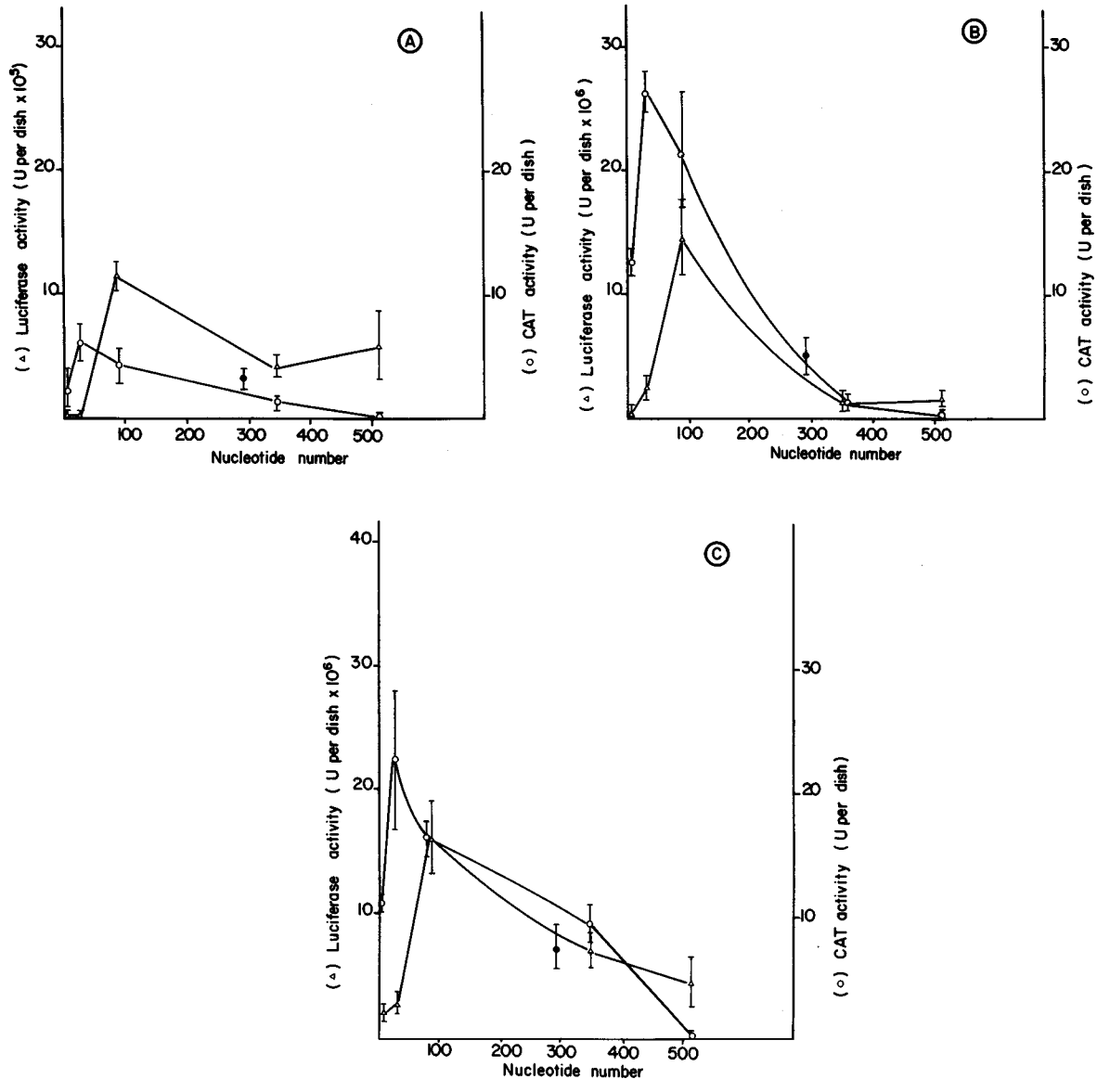

FIG. 3. Effect of spacer length on the expression of luciferase and CAT cistrons in CHO cells. The enzymatic activities are the means ± SEM from the independent dishes. (A) Spacers alone, (B) spacers followed by the poliovirus IRES, and (C) spacers followed by the SUR IRES (△) luciferase activity and (○) CAT activity in constructs having spacers of variable length, (●) CAT activity in the constructs having as spacer a 293-nucleotide DNA fragment containing the rabbit WAP gene terminator.

The Translation Rate of the Cistrons Is Not Strictly Related to mRNA Concentration

The constructs having spacers of variable length were transfected into CHO cells and the corresponding mRNAs were analyzed by Northern blot assay. In all cases, the concentration of total RNA was checked by illuminating the nylon membrane after the transfer from the gel. The concentration of total RNA was the same in all lanes and the rRNAs were not at all degraded.

Results shown in Fig. 4 indicate with no ambiguity that the amount of mRNA containing the luciferase and CAT cistrons was not related to the translation rate of both cistrons. The mRNA concentration was lower with the shortest and the longest spacers.

With plasmids containing the 293–344-nucleotide spacers, the concentration of the mRNA was still high whereas the corresponding CAT activity was very low. This result indicates that the use of IRES to express the cistrons of bicistronic mRNAs is more complex than generally imagined.

Reducing the Length the First Cistron Increases Translation of the Second Cistron

A certain number of mRNA have several very short cistrons in their 5′ UTR before the AUG codon

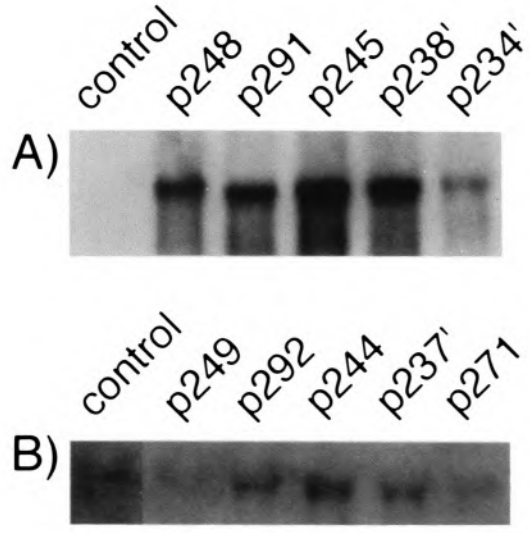

FIG. 4. Effect of spacer length on the concentration of mRNA. Transfections were carried out in CHO cells. Two days later, total RNA was extracted and submitted to Northern blot analysis. 10 µg of total RNA was used and hybridization was carried out using luciferase ^{32}P-labeled cDNA as probe. Autoradiography was of 4 days. Control was nontransfected cells. (A) Plasmids with the SUR IRES and (B) plasmids with the poliovirus IRES.

used to start translation of the mRNA. This is particularly the case for the 5′ UTR of the RSV RNA (8). Some of the short cistrons are translated and the presence of these short cistrons seems to favor translation of the major cistron.

In a previous work (1) it was observed that, in bicistronic mRNAs, the second cistron (the bovine GH cDNA) preceded by the SUR IRES was better expressed when the first cistron was shorter. The first cistrons used in this study were the human GH cDNA (0.6 kb) or the firefly luciferase cDNA (1.6 kb). On the other hand, it is not known if the proximity of the cap and the IRES influences the translation rate of the following cistron.

To tentatively evaluate this point, the second cistron was introduced after a short open reading frame. To reach this goal, the constructs depicted in Fig. 5A were done. The luciferase gene was interrupted after 110 bp by a UAG codon. The CAT gene preceded by a 100-bp spacer was added after the UAG codon. Results shown in Fig. 5B indicate that the 100-nucleotide spacer allowed a low but significant CAT activity. The CAT gene was better expressed when it was added after 110 bp of the luciferase gene than after the complete gene. The CAT activity represented about 12% of that obtained with the corresponding monocistronic vector (p232).

The poliovirus IRES (p273′) did not enhance CAT activity, whereas the SUR IRES (p274) was highly efficient (Fig. 5B). With the last IRES, up to 65% of

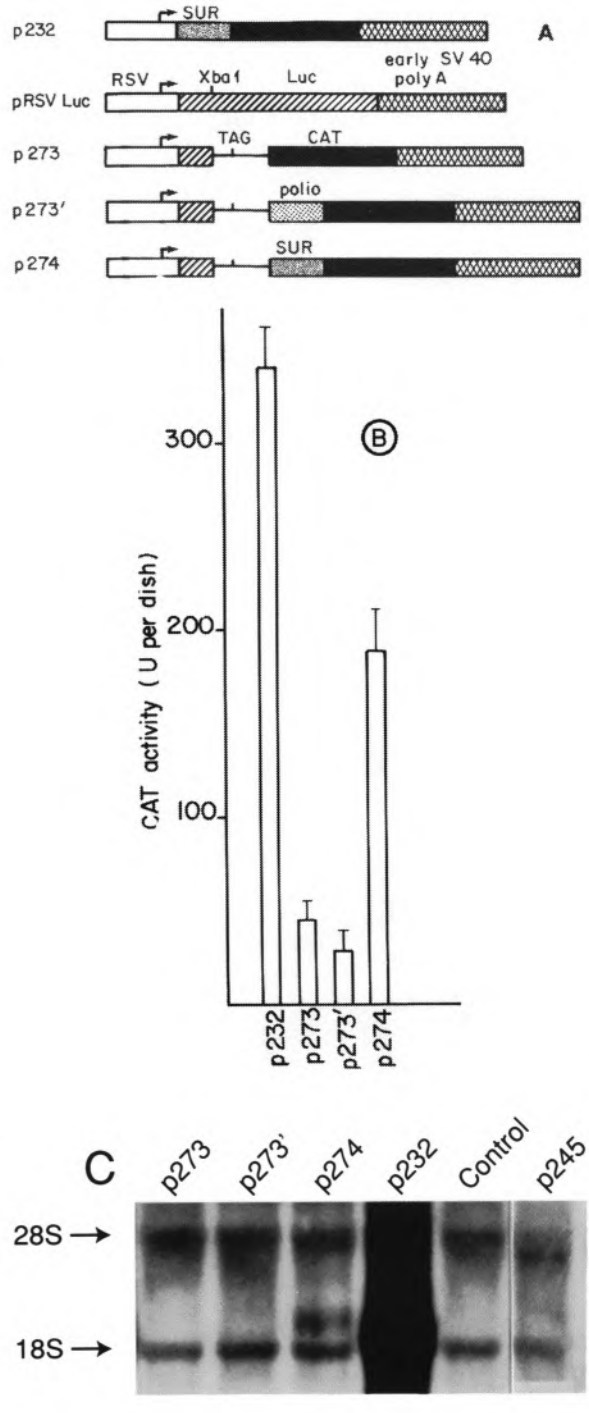

FIG. 5. Effect of the IRES on the translation of the CAT cistron inserted in the upstream part of the luciferase cistron and after a termination codon. The CAT cistron was introduced in the *Xba*I site of the luciferase gene. A short linker (110 nucleotides) containing a termination codon was added directly before the CAT gene (p273) or before the poliovirus (p273′) or the SUR (p274) IRESs. The CAT activity was measured in the CHO cell extracts. Results are the means ± SEM from three independent dishes. (A) Schematic representation of the constructs and (B) CAT activity. (C) Northern blot analysis.

the CAT activity of the corresponding monocistronic vector was obtained.

Northern blot analysis revealed that the CAT mRNA was abundant with the control plasmid p232. The mRNA was considerably reduced with the three other gene constructs (Fig. 5C). This may be attributed to the presence of the termination codon in the 5′ P part of the luciferase mRNA. Indeed, it has been shown that the presence of termination codon in a short distance of the initiation codon destabilizes the mRNA, most likely by preventing its normal transfer from the nucleus to the cytoplasm (9).

In the construct containing the SUR IRES, a small but significant amount of mRNA was present. A previous study has shown that SUR favors accumulation of the associated mRNAs in various cell types. However, this effect was very weak in CHO cells (2). One possible explanation is that, in the absence of IRES, the mRNAs containing a UAG codon were destabilized. The poliovirus IRES might be not of sufficient efficiency in this position to favor translation. On the contrary, the SUR IRES was able to reinitiate translation leading to a partial stabilization of the mRNA.

These data support the idea that an IRES, or at least some of them, are more efficient when they are present after a short cistron or are closer to the cap.

Another point is particularly striking. The amount of mRNA was generally much lower with bicistronic than with monocistronic constructs. On the other hand, the level of CAT activity was not related to the amount of mRNA (Fig. 5B). This observation is compatible with the idea that IRESs are translation stimulators having a potency that depends on their position in the mRNA.

IRES Introduced Within a Translated Cistron Initiates Translation of the Second Cistron

In a preliminary experiment not depicted here, we introduced the bovine growth hormone (bGH) cDNA added after the SUR IRES in the *Asp*1 site of the human growth hormone gene (hGH), located in the last exon, before the termination codon. To our surprise, we observed that the bGH cDNA was quite well expressed. A similar vector containing the human erythropoietin cDNA instead of the bGH cDNA expressed the erythropoietin coding sequence in transfected cells and in transgenic animals (18). These observations suggested that an IRES can interrupt translation of an mRNA when it is introduced into its coding region. In order to check if this hypothesis is valid, gene constructs containing both reporter genes separated by spacers allowing the CAT gene to be or not be in the same reading frame as the luciferase gene were prepared. For that purpose, the CAT gene was introduced into the *Eco*RV site of the luciferase gene with three different spacers, allowing translation of the CAT mRNA in the three possible reading frames of the luciferase mRNA. In the same constructs, the poliovirus and the SUR IRESs were added (Fig. 6A).

As could be expected, only one of the three vectors having one of the spacers but no IRES (p251, p253, and p255) led to the synthesis of CAT (p253). In this case, the CAT activity most likely resulted from a fusion protein containing the beginning of luciferase and the complete CAT.

The poliovirus and the SUR IRESs were added into the vectors generating the plasmids p251′, p253′, and p255′ on one hand and p250′, p252′, and p254′ on the other (Fig. 6A). As expected, the IRES allowed a better expression of the CAT gene (Fig. 6B). This effect was obtained with the three reading frames.

Northern blot analysis revealed that the concentration of the CAT mRNA was similar with the different constructs. On the other hand, the different mRNAs had the expected size (Fig. 6C). This suggests that the poliovirus and the SUR IRESs enhanced CAT activity essentially by stimulating translation of the cistron.

DISCUSSION

The IRES are extensively used to express several cistrons from a single mRNA. The mechanism of action of IRES is not fully understood, and this may explain why a certain number of experimenters obtained unreliable results to express cistrons of bicistronic mRNAs.

It is striking that the distance between the end of the first cistron and the beginning of the IRES has a strong influence on the expression of the second cistron and to some extent of the first cistron.

The activity of enzymes coded by both cistrons was not strictly related to the concentration of the mRNA. Somewhat unexpectedly, the cistrons were poorly expressed when the spacers were the shortest. One may imagine that, in this condition, the ribosomes that finished translation of the first cistron do not have enough space to be properly released and hampered translation of the second. This may lead to a low translation rate of the mRNA and consequently to its destabilization. This could explain why the concentration of mRNA was lower with the spacers containing 8–9 nucleotides than with those containing 86–89 nucleotides.

The bicistronic mRNAs containing long spacers before the IRES were very poorly translated. The mRNA containing the longest spacers (516–517 nucleotides) were present at a low concentration as

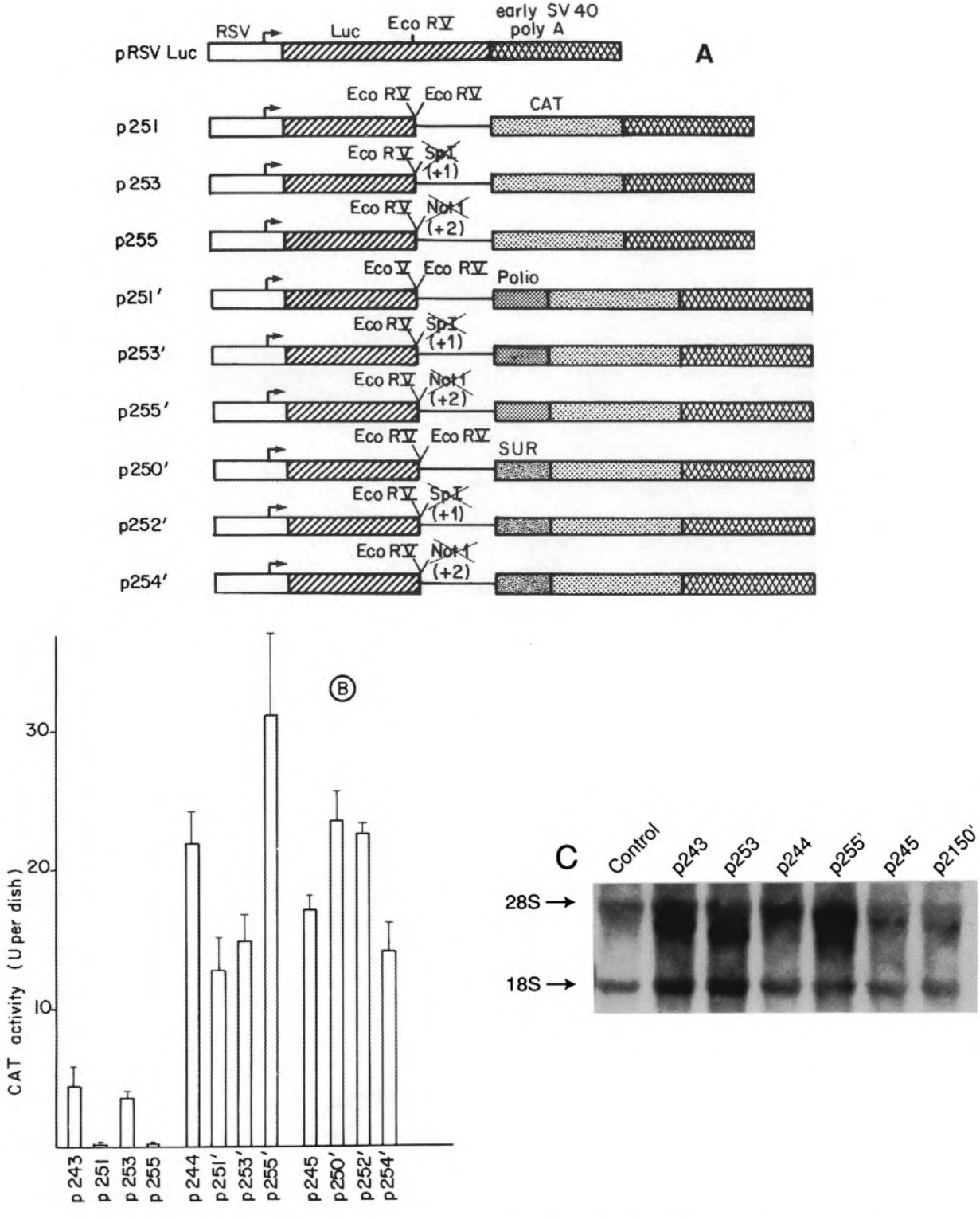

FIG. 6. Effect of the IRES on the translation of the CAT gene inserted in the luciferase gene without a prior termination codon. The CAT gene was inserted in the *Eco*RV site of the luciferase gene. Linkers were added before the CAT gene to put this gene in the three reading frames of the luciferase coding sequence. (+!) and (+2) means that one and two additional nucleotides were present, respectively, in the linker to shift the reading frame. The poliovirus and the SUR IRES were added or not before the CAT gene. The CAT activity was measured in the CHO cell extracts. Results are the means ± SEM from three independent dishes. (A) Schematic representation of the constructs and (B) CAT activity. (C) Northern blot analysis.

though their low translation rate resulted in their destabilization. The fact that the pattern of expression as a function of spacer length was essentially the same for the mRNA containing or not containing an IRES may suggest that IRESs are essentially translation stimulators. These data suggest that the mechanism of action of IRES is more complex than generally admitted. A possible nonphysiological interference of the spacers with the IRES, although unlikely, cannot be completely ruled out. This point was discussed in other articles (3,11). In a recent review, Kozak (16) also concluded that the notion of IRES might be not fully established.

In practical terms, the data depicted here indicate that the distance between the end of the first cistron and the beginning of an IRES should contain about 60–100 nucleotides to allow an optimum expression of both cistrons.

The fact that the IRES were able to translate the following cistron irrespective of the reading frame of the cistron in which they were introduced is also of interest. The mechanism of action of this phenomenon is not known. In practical terms, it indicates that a cistron preceded by an IRES may be added at any site in the mRNA and be efficiently translated.

When the IRESs from poliovirus and HTLV-1 were added after a truncated first cistron, the translation of the second cistron was not similar. For unknown reasons, the HTLV-1 IRES stimulated strongly the expression of the second cistron but the poliovirus IRES did not. After a complete cistron, both IRESs showed essentially the same activity. One possible explanation is that the HTLV-1 IRES, which is naturally present in a retroviral mRNA, may cooperate with the cap located 110 nucleotides upstream. On the contrary, the poliovirus IRES, which is known to be active even in the absence of functional cap in the viral mRNA, may not cooperate with factors possibly associated with the cap. In practice, these data suggest that the different IRESs are not strictly equivalent. They seem to work with somewhat different mechanisms. The choice of an IRES to express efficiently a cistron should be done accordingly.

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