KOR. JOUR. MICROBIOL. June, 1988 p. 106-112 Vol. 26, No. 2

The Abundant Presence of Nonpolyadenylated SV40 Late 19S Spliced RNA in the Nucleus of Monkey Cell

Jung-Hye Roe[1] and Janet Mertz[2]

[1]*Department of Microbiology, College of Natural Sciences, Seoul National University*

[2]*McArdle Laboratory for Cancer Research, University of Wisconsin-Madison*

Madison, Wisconsin 53706

Poly A tail을 결핍한 Simian Virus 40 spliced RNA의 세포내 분포

노정혜[1]·Janet Mertz[2]

[1]*서울대학교 자연과학대학 미생물학과*

[2]*McArdle Laboratory for Cancer Research, University of Wisconsin, Madison*

ABSTRACT: We have examined the structures and cellular distributions of the SV40 late RNAs present in monkey cells at late times after infection. One particular RNA species, spliced at residue 373 (373-RNA), was found to be as abundant as the major late 16S RNAs. This result was unexpected since previous reports showed that the molecular ratio of the 373-spliced 19S RNA to 16S RNA is approximately 0.1 among either cytoplasmic polyadenylated or polysomal viral RNAs. Both sedimentation and electrophoretic analysis indicated that the 373-RNA was approximately 16S to 19S in size. Therefore, it was not a splicing intermediate or the product of premature termination of transcription within the late leader region. Whereas most SV40 late 16S RNA is polyadenylated and located in the cytoplasm, the majority of 373-RNA was found to lack poly A, and be located in the nucleus.

KEY WORDS □ SV40 late RNAs, Poly A (-) spliced RNA, mRNA transport, S1 nuclease mapping.

Late in the lytic cycle of infection of monkey cells, SV40 produces two classes of late RNAs, 16S and 19S in size, which encode the virion proteins VP1 and VP2/3, respectively. These RNAs are made by differential splicing of the primary nuclear transcripts and exhibit heterogeneity in their 5'ends as well as splice sites utilized in processing (Ghosh *et al.*, 1978; Reddy *et al.*, 1978; Ghosh *et al.*, 1982; Somasekhar and Mertz, 1985a, b; see Figure 1). The mechanism of selection of splice site is not yet known. The relative abundances of the various species of spliced late RNAs present in the cytoplasm of SV40-infected monkey cells has been determined for both polysomal and polyadenylated RNA (Ghosh *et al.*, 1978; Reddy *et al.*, 1978; Ghosh *et al.*, 1982; Barkan and Mertz, 1984; Somasekhar and Mertz, 1985a, b), with the ratio of 16S to 19S cytoplasmic, polyadenylated RNA being approximately four (Barkan, 1983).

In this report, we show that the steady state level of the SV40 late spliced 19S RNAs is similar in amount to that of the late 16S RNAs in the whole cell. However, whereas most of the 16S RNA is polyadenylated, much of the 19S-spliced RNA lacks poly A and, therefore was overlooked in previous analysis. This unexpected finding indicates that the choice of splice sites utilized in processing affects not only the proteins encoded by the processed RNA but also its subsequent metabolism.

MATERIALS AND METHODS

Purification of RNA from Infected Cells

Freshly confluent monolayers of CV-1P cells were infected as described previously (Mertz and Berg, 1974) with SV40 strain wild-type (wt) 830 at a multiplicity of infection of approximately 20 pfu per cell and were incubated for 48h in a 37 °C CO_2 incubator in DMEM (GIBCO) containing 2% fetal bovine serum. For the preparation of whole cell RNA, the cells were rinsed with Tris-buffered saline lacking Ca^{2+} and Mg^{2+} (TD buffer) and were lysed directly in the dish by the addition of 200 μg/m*l* proteinase K in 50 mM Tris (pH 7.4), 10 mM EDTA and 1% SDS. For the preparation of nuclear and cytoplasmic RNA, the cells were harvested with a rubber policeman into TD buffer, washed, and lyzed by incubation at 0 °C in 0.5% NP-40, 5mM vanadyl-ribonucleoside complex (VRC) for 5 min with occasional gentle vortexing. The nuclei were pelleted by centrifugation at 2K rpm for 5 min at 4 °C. The supernatant (= cytoplasm) was mixed with an equal volume of 2 × proteinase K solution (1 × proteinase K solution equals 250 μg/m*l* proteinase K in 0.1 M Tris (pH 7.5), 12 mM EDTA, 0.15 M NaCl, and 1% SDS). The nuclei were washed in 0.5% NP-40, 10 mM VRC, and the supernatant was added to the cytoplasmic solution containing proteinase K. Finally, the nuclei were resuspended in the same buffer, and lyzed by the addition of an equal volume of 2 × proteinase K solution followed by vortexing. Each lysate (whole cell, nuclear, and cytoplasmic) was incubated at 37 °C for 1-2 hrs, extracted with phenol:chloroform:isoamylalcohol until the interphase was clean, extracted with chloroform: isoamylalcohol, and precipitated with ethanol. The whole cell and nuclear RNA samples were then treated with DNase I (5 μg/plate; Worthington) in 10 mM Hepes (pH 7), 5 mM $CaCl_2$, 10 mM $MgCl_2$, 1 mM DTT, and repurified as above. After precipitation with ethanol, the RNA in each pellet was resuspended in 10 mM Tris (pH 7.4), 1 mM EDTA and stored at -20 °C.

RNA was further fractionated through oligo (dT)-cellulose column. We followed the procedures of Aviv and Leder (1972) using 0.2 m*l* of oligo(dT)-cellulose (Collaborative Research) in a 1 m*l* syringe.

S1 Mapping Analysis

The probe used for S1 mapping, depicted in Figure 1, consists of the SV40 sequences from nt. 334 (an NcoI site) to nt.770 (an EcoRV site) linked to sequences from pBR322 DNA (EcoRV to Eco RI). This 622 bp long DNA fragment was isolated by complete digestion with EcoRI and partial digestion with NcoI of a recombinant plasmid in which wt SV40 DNA and pBR322 had been joined at their EcoRV sites. Restriction enzymes were purchased from New England Biolabs. After gel purification, the DNA probe was labeled at its 3 'end with the Klenow fragment of DNA poly-

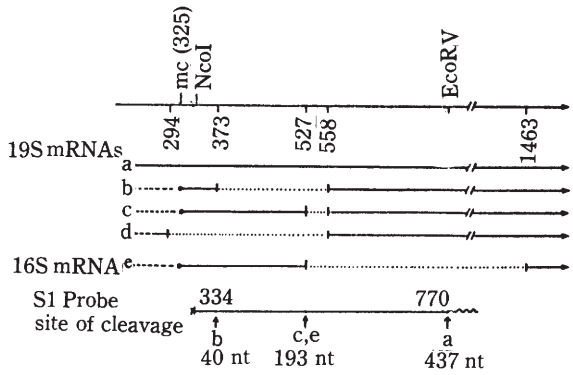

Fig. 1. *Structures of the late region of the SV40 genome, the abundant mRNAs it encodes, the probe used for S1 mapping.*

The top line depicts part of the SV40 genome transcribed at late times after infection. The nucleotide numbering is according to Tooze (1982). The structures of the various abundant late mRNA species are indicated by the lines labeled a-e (Ghosh *et al.*, 1978). Their spliced out introns are denoted by dotted lines and their heterogeneous 5' ends by dashes. mc denotes the location of the major cap site at nucleotide residue 325. The probe used for S1 nuclease mapping was 3' end-labeled at residue 334. The wavy line indicates the pBR322 sequences present in the probe. 437 and 40 nucleotide long labeled DNA is protected from S1 nuclease digestion by RNA a and b, respectively, while 193 nucleotide long fragment is protected by RNAs c and e.

merase I (Promega Biotech) and α-^{32}P-dCTP.

Each RNA sample and a molar excess of 3′ end-labeled probe DNA were resuspended in 5 μl of 80% formamide, 40 mM Pipes (pH 6.8), 400 mM NaCl, 1 mM EDTA. After incubation at 85 °C for 10 min, each mixture was incubated at 51.5 °C for 18-20 hs, followed by treatment with S1 nuclease at 22 °C for 1.5 hs in 280 mM NaCl, 30 mM NaOAc (pH 5.5), 1 mM ZnSO4, 10 ug/m*l* denatured calf thymus DNA. The undegraded DNA was precipitated with ethanol, dissolved in alkaline loading buffer (80% formamide, 20 mM NaOH, 10 mM EDTA, 0.25% each of xylene cyanol and bromophenol blue), incubated at 100 °C for 3 min, and analyzed by electrophoresis in a 10% polyacrylamide gel containing 7 M urea. The amount of DNA in each band was determined by quantitative autoradiography. All the reagents used are of reagent grade from Sigma and S1 nuclease is a kind gift from Dr. Jeffrey Ross (University of Wisconsin).

Northern Blot Analysis of RNA

RNA samples obtained from whole cell and nucleus of SV40-infected monkey cells were denatured with glyoxal and DMSO (Maniatis *et al.*, 1982), electrophoresed in 1% agarose gel, and transfered to nitrocellulose paper. The blot was then analyzed specifically for late 19S RNA by hybridization as described by Thomas (1983) with an NcoI (nt.555)-to-HindIII (nt.1046) restriction fragment of SV40 that had been radiolabeled by nick-translation (Rigby *et al.*, 1977).

RESULTS

Aloni and colleagues (Hay *et al.*, 1982) have postulated the existence of transcription pause and termination sites mapping within the late leader region of SV40. In possible agreement with this hypothesis, Mertz (unpublished data) observed in *Xenopus* oocytes injected with SV40 DNA the appearance of small RNAs with 3′ends mapping to nucleotide residue 373-374. To look for such small RNAs in SV40-infected monkey cells, we performed a series of S1 nuclease mapping experiments with a probe 3′end-labeled at residue 334 to enable detection of all SV40-specific late RNA species with 3′ ends or discontinuties mapping within the major late leader region (Figure 1).

Initially, whole cell, nuclear, and cytoplasmic RNA samples were analyzed (Figure 2). As can be seen in Figure 2, lane c, we observed that viral RNA with a discontinuity around residue 373 (373-RNA) is as abundant in whole cells at late times after infection as is the late major 16S RNA. This result was unexpected since previous reports indicated that the molecular ratio of the 373-spliced 19S RNAs to the 526-spliced 16S RNAs is approximately 0.1 among either polyadenylated cytoplasmic or polysomal viral RNA (Barkan, 1983; Somasekhar & Mertz, 1985b). Therefore, the majority of the 373-RNA detected

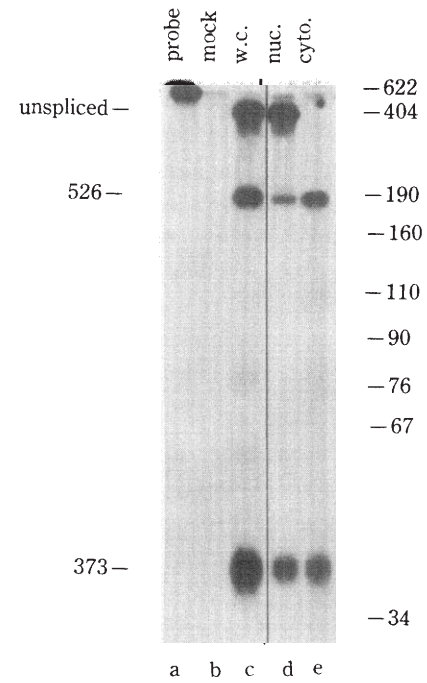

Fig. 2. *S1 mapping of the SV40 late RNAs.*
RNA was purified from whole cells (w.c.) or the nucleus (nuc.) and cytoplasm (cyto.) of cells at 48 hs after infection, or from uninfected cells (mock). The numbers on the left indicate the nucleotide residue numbers of donor splice sites of the RNA species from which the corresponding protected DNA fragments were generated. The locations of molecular weight markers (MspI-digested pBR 322 DNA) are shown on the right.

in our assay must have been something other than viral messenger RNA.

Several possibilities were considered as to the identify of this abundant 373-RNA. First, this RNA might be small RNA with a 3′ end at or near nucleotide residue 373 produced either as an intermediate in splicing (i.e., an unligated exon) or by premature termination of transcription. Alternatively, 373-RNA might be nt.373-to nt. 558-spliced 19S RNA or nt.373-to nt.1463 spliced 16S RNA. The last of these possibilities was excluded on the basis of the failure to detect such a species in SV40-infected whole cell RNA at even the 1% level by the modified primer extension technique (P.J. Good, Personal Communication).

To examine the cellular distribution of this novel viral RNA species, SV40-infected cells were fractionated into nuclear and cytoplasmic components and the RNAs in each component were separately purified and S1 mapped as above (Figure 2, lanes d and e). Densitometric analysis of autoradiograms of gels similar to the one shown in Figure 2 indicated that whereas only one fourth of 526-RNA which is mostly 16S RNA was located in the nucleus, approximately 70% of the 373-RNA was in the nucleus (Table 1). The exclusive distribution of unspliced viral RNA to the nucleus (Figure 2) and the 18S and 28S ribosomal RNAs to the cytoplasm (data not shown) excluded the possibility of cross-contamination between the nuclear and cytoplasmic RNA samples.

To determine whether the abundant 373-RNA contained any poly A, we fractionated the RNA samples further by chromatography through columns of oligo(dT)-cellulose prior to mapping with S1 nuclease. An autoradiogram of the data obtained from one such experiment is presented in Figure 3. Table 1 summarizes the data obtained

Table 1. *Fractional Distribution of SV40 Late RNAs*

	Whole Cell		Nucleus		Cytoplasm	
	A+	A−	A+	A−	A+	A−
373-RNA (19S)	0.3	0.7	0.1	0.6	0.2	0.1
526-RNA (16S)	0.7	0.3	0.1	0.15	0.6	0.15

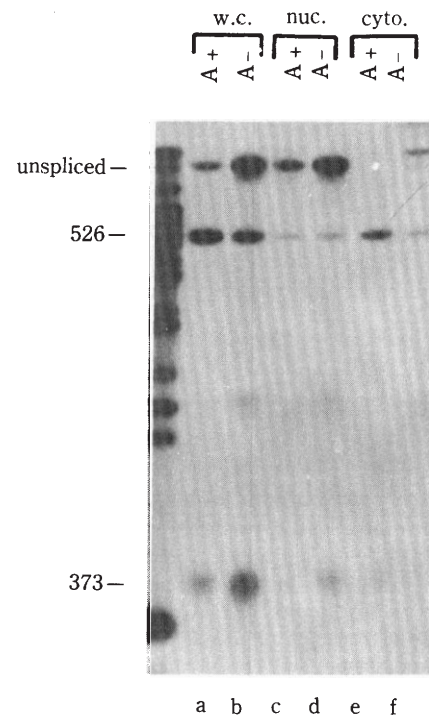

Fig. 3. *S1 mapping of viral RNAs fractionated by oligo (dT)-cellulose column chromatography.*

from the analysis in this manner of viral RNA samples purified independently from several batches of infected cells. These data indicate that, whereas the majority of the 373-RNA present in cells was devoid of poly A, the small percentage of it that was polyadenylated was largely found in the cytoplasm. On the other hand, most of the 526-RNA (16S) contained poly A and was located in the cytoplasm. As expected, the majority of the unspliced RNA lacks ploy A and was also located in the nucleus.

To determine whether the abundant 373-RNA was small in size, the nuclear and cytoplasmic RNA samples were separately sedimented through gradients of sucrose and the viral RNA present in each fraction was analyzed by S1 mapping as above. The data shown on Figure 4 indicate that the 373-RNA present in the cytoplasm was message-sized, co-sedimenting approximately with the 16S to 19S RNAs. Analysis of the nuclear sample yielded essentially identical results (data not shown).

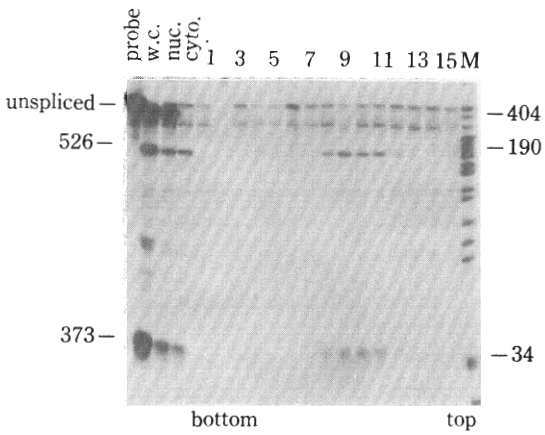

Fig. 4. *S1 mapping of viral RNA fractionated by sedimentation through sucrose.*
RNAs were heated for 5 min at 65°C, layered onto a 15-30% gradient of sucrose and sedimented in SW6ϕ rotor at 32 K rpm for 18 hs at 4°C. 28-30 fractions (0.15 m*l* each) were collected from the bottom of the gradient. A portion of the fractionated RNA was analyzed by S1 mapping as in Figure 2. Ribosomal 28S, 18S, and 5S RNAs co-sedimented with fractions 3, 8, and 13, respectively. The molecular weight markers (M) are MspI-digested pBR322 DNA.

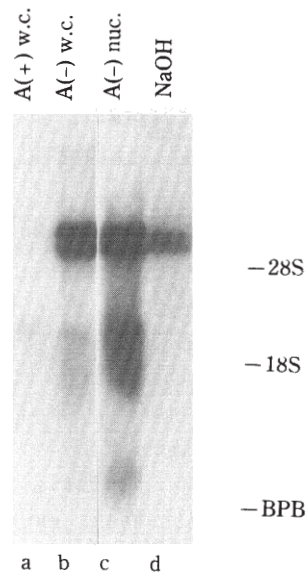

Fig. 5. *Northern blot analysis of viral late 19S RNAs.*
RNA samples were electrophoresed and blotted as described in the text. A sample treated with 1N NaOH was analyzed in parallel as a negative control (lane d). Ribosomal 28S and 18S RNAs and the front of the marker dye bromophenol blue (BPB) ran as indicated on the right.

Lastly, northern blot analysis was performed using ^{32}P-labeled probe specific for 19S RNA (Figure 5). The probe used consists of intron sequences of 16S mRNA and hence does not detect spliced 16S RNA. Alkaline-treated samples were run in parallel as a control for non-RNA bands (Figure 5, lane d). The results showed that whereas poly A(+) 19S RNA from whole cells migrated as a distinct band (lane a) poly A(-) RNA from either whole cell or nuclei migrated as a smear, ranging in size from approximately 1.0 to 2.0 kb in length (lanes b and c). The dark bands near the top of the gel were obviously not from RNA since they were resistant to treatment with alkali (lane d). This result unequivocally indicates that poly A(-) 373 RNA is not small in size, and cannot be a product of transcriptional pausing or premature termination in the late leader region of SV40. The structure of this RNA needs to be more accurately determined in order to elucidate the function and the metabolism of this RNA. The 5′ and 3′ end mapping as well as splicing pattern analysis is currently under investigation.

DISCUSSION

We report here the existence of an abundant RNA species in the nucleus of SV40-infected cells which, although produced similar to the major polyadenylated cytoplasmic late 19S RNA by splicing from 373, lacks a poly A tail. The possibility that this novel RNA species was generated by degradation during purification of the RNA is unlikely for several reasons. First, although the nuclear and cytoplasmic samples were purified identically in parallel after cell fractionation, the fraction of the 373 RNA that was non-polyadenylated was quite different between the two (see Table 1). Second, the size distribution of 19S RNA as shown both by sucrose gradient sedimentation (Fig. 4) and gel electrophoresis (Fig. 5) was non-random; in other words, not all size classes of RNAs were represented. The presence of non-polyadenylated SV40 late RNA in the nucleus of

infected monkey cells has been known for some time, with the majority of it having been reported to be unspliced RNA (Villarreal and Jovanovich, 1980; Lai *et al.*, 1978). Lai *et al.* (1978) have reported that unspliced 19S RNA is scarce among non-polyadenylated nuclear viral RNA, while adenylated nuclear RNA contains significant amounts of spliced species. This observation led to the hypothesis that polyadenylation of SV40 late message occurs either prior to or concomitantly with splicing. Our work clearly shows that there is a significant amount of spliced non-polyadenylated 19S RNA in the nucleus, consistent with the report of Villarreal and Jovanovich (1980). Therefore, it would appear that splicing and polyadenylation of the SV40 late transcription may occur in either order.

The postulation of pathways for the generation of non-polyadenylated 373-RNA must await the structure determination of this RNA. The heterogeneity in the size of this RNA could have resulted from any of the following; heterogeneities in the 5′ end, 3′ end, as well as the intron length. However, regardless of the mechanism of formation of this RNA localized in the nucleus, it is reasonable to assume that the absence of the poly A segment may be responsible for its accumulation in the nucleus. This mechanism, if true, may be one way by which the cell regulates the level of translatable message present in the cytoplasm.

적 요

SV40 바이러스가 원숭이 세포에 감염한 후기에 만들어지는 바이러스 RNA들의 구조와 세포내 분포를 조사하였다. SV40 genome상 373번 잔기에서 splicing이 일어난 특정한 RNA 종류(373-RNA)는 감염후기의 주된 RNA인 16S RNA만큼 많은 양이 축적되는 것을 S1 nuclease mapping결과 알게 되었다.

Sucrose gradient상에서의 침강속도와 전기영동 속도로 볼 때 373-RNA는 약 16-19S의 크기를 가지는 것으로 밝혀졌다. 따라서 이 RNA는 splicing 과정의 부산물이거나 전사의 미성숙한 종결과정에서 생겨나는 RNA가 아님을 추론할 수 있다. 16S RNA가 대부분 poly A 꼬리를 갖고 세포질에 편재해 있는데 반해, 373-RNA는 대부분이 poly A 꼬리를 갖지 않고 핵에 편재해 있었다.

REFERENCES

1. Aviv, H. and P. Leder, 1972, Purification of biologically active globin messenger RNA by chromatography on oligo thymidylic acid-cellulose. *Proc. Natl. Acad. Sci.* **69,** 1408-1412.
2. Barkan, A., 1983, Ph. D. Thesis, University of Wisconsin, Madison, Wisconsin.
3. Barkan, A. and J. Mertz, 1984, The number of ribosomes on simian virus 40 late 16S mRNA is determined in part by the nucleotide sequence of its leader. *Mol. Cell. Biol.* **4,** 813-816.
4. Ghosh, P.K., V.B. Reddy, J. Swincoe, P. Lebowitz, and S.M. Weissman 1978, Heterogeneity and 5′-terminal structures of the late RNAs of simian virus 40. *J. Mol. Biol.* **126,** 813-846.
5. Ghosh, P.K., M. Piatak, J.E. Mertz, S.M. Weissman, and P. Lebowitz, 1982, Altered utilization of splice sites and 5′ termini in late RNAs produced by leader region mutants of simian virus 40. *J. Virology* **44,** 510-524.
6. Hay, N., H. Skolnik-David, and Y. Aloni, 1982, Attenuation in the control of SV40 gene expression. *Cell* **29,** 183-193.
7. Lai, C.J., R. Dhar, and G. Khoury, 1982, Mapping the spliced and unspliced late lytic SV40 RNAs. *Cell* **14,** 971-982.
8. Maniatis, T., E.F. Fritsch. and J. Sambrook, 1982, Molecular Cloning, pp 200-201. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
9. Mertz, J. and P. Berg, 1974, Defective simian virus 40 genomes: isolation and growth of individual clones. Virology **62,** 112-124.

10. Reddy, V., P. Ghosh, and S. Weissman, 1978, Gaps and duplicated sequences in the leaders of SV40 16S RNA. *Nucleic Acids Res.* **5,** 4193-4213.
11. Rigby, P.W.J., M. Dieckmann, C. Rhodes, and P. Berg, 1977, Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J. Mol. Biol.* **113,** 237-251.
12. Somasekhar, M. and J. Mertz, 1985a, Sequences involved in determining the locations of the 5′ ends of the late RNAs of simian virus 40. *J. Virol.* **56,** 1002-1013.
13. Somasekhar, M. and J. Mertz, 1985b, Exon mutations that affect the choice of splice sites used in processing the SV40 late transcripts. *Nucleic Acids Res.* **13,** 5591-5609.
14. Thomas, P., 1983, Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. *Methods Enzym.* **100,** 255.
15. Tooze, J. (ed), 1980, DNA Tumor Viruses. Molecular Biology of Tumor Viruses, 2nd. ed., part2. Cold Spring Harbor Laboratory, CSH, N.Y.
16. Villarreal, L.P., and S. Jovanovich, 1980, Leakage of nuclear transcripts late in simian virus 40-infected CV1 cells. *J. Virol.* **36,** 595-600.

(Received Apr. 30, 1988)