The murine 2-5A synthetase locus: three distinct transcripts from two linked genes

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ABSTRACT

The cloning of cDNAs encoding murine 2-5A synthetase has identified three related transcripts, represented by a previously described cDNA clone, L3 and two novel cDNAs, L1 and L2. L1 contains an open reading frame coding for a protein that shows 70% conservation at the amino acid level when compared to the protein predicted to be encoded by L3. L2 recognizes an IFN-induced transcript 600-bp larger than the L3 transcript. These three cDNAs map to a cosmid, cII, containing two murine 2-5A synthetase genes, ME12 and ME5/ME8, situated in a head-to-tail orientation separated by approximately 8 kb. Southern analyses of ME12 and ME5/ME8 using L3, L1-specific and L2-specific probes indicate that these genes have a similar organization. cII was transiently and stably transfected into CV-1 cells. When treated with interferon, the transfected cells produced mature, murine 2-5A synthetase transcripts identified using L3 and L2-specific probes. Thus all transcripts present in IFN-treated mouse cells which are recognized by the available murine 2-5A synthetase cDNA probes map to an approximately 40 kb region of the mouse genome containing two 2-5A synthetase genes.

INTRODUCTION

The 2-5A system regulates RNA stability in virus-infected, interferon (IFN)-treated cells but it is likely to have a wider role in control of normal cell growth (for reviews see Refs. 1,2). The central molecules of this system, 2-5A [pppA(2′p5′A)$_n$, n=1 or more] are produced by IFN-inducible, double stranded (ds) RNA-dependent 2-5A synthetase (3) and are required for activation of 2-5A-dependent RNase, an enzyme that cleaves single-stranded viral and cellular RNA on the 3′-side of UpNp doublets (1,2). 2-5A is unstable in cells because it is degraded by a 2′,5′-phosphodiesterase and also by phosphatases which ensures that the activation of the RNase is transient (4, 5). Biochemical studies of 2-5A synthetases from tissues and cell lines from various species demonstrated the presence of different sizes of 2-5A synthetase (6−13). Cloning experiments by several groups have identified cDNAs for 1.6 kb and 1.8 kb mRNAs which are derived from a single human gene by differential splicing and encode 40 kd and 46 kd 2-5A synthetase proteins respectively; these two proteins are identical in their first 346 residues but have unique C-terminal ends (14−18). Immunoprecipitation and immunoblotting studies detected these proteins and two, additional larger molecular weight synthetase proteins of 69 kd and 100 kd (19, 20). Each of the four proteins has 2-5A synthetase activity but the optimum concentration of dsRNA required for enzyme activation is different for the individual enzymes (13, 19, 21). The average length of oligomers produced by different forms of the enzyme are also different (21). 2-5A synthetase proteins have been localized on microsomes (100 kd), in nuclei (40 kd, 46 kd, 67 kd) and on membrane structures (69 kd) (19, 20, 22). Furthermore, different forms of the proteins display cell-specific expression and differences in the kinetics of IFN-induction (19, 20, 21). The 69 kd and 100 kd proteins react with antibodies against a peptide common to the human 40 kd and 46 kd proteins therefore it is expected that there will be some similarity at the RNA level between the small (40 kd and 46 kd) and large (69 kd and 100 kd) synthetases (19, 20).

The human 2-5A synthetases form a complex, multienzyme system which undoubtedly has an analogue in the mouse. Large and small molecular weight 2-5A synthetase mRNAs and proteins have been detected in mouse cells (10, 20). To date, only cDNAs for a small (40 kd) mouse 2-5A synthetase protein have been cloned (23, 24, 25). However, whereas only one human 2-5A synthetase gene has been isolated, two murine 2-5A synthetase genes have been described, each containing a functional, IFN-inducible promoter (25). We were interested in exploring the possibility that these genes give rise to large molecular weight mRNAs encoding large (69 kd or 100 kd) mouse 2-5A synthetases in addition to small molecular weight mRNA encoding the small mouse synthetase protein(s). Here we describe two novel mouse 2-5A synthetase cDNAs that map to these genes. One of these cDNAs may partially encode a large synthetase protein. We also report the isolation and mapping of a cosmid containing both murine 2-5A synthetase genes. Transfection assays employing this cosmid indicate that it will be a valuable tool for investigating the origin of large mouse 2-5A synthetase transcripts and proteins.

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MATERIALS AND METHODS

cDNA and genomic library screening

Overlapping cDNA clones J4 and N1A were isolated from a λgt11 cDNA library prepared from IFN-induced mouse JLSV9R cells under screening conditions that have been previously described (23). Another cDNA, clone B, was isolated from a library prepared from synchronized mouse embryo fibroblasts reaching the S-phase (22). These cDNAs were restriction mapped and sequenced using standard techniques (31). Clones B and J4 were contained within the larger cDNA N1A which, for convenience, was renamed L1. L2 was isolated along with clone L3 from a λgt11 cDNA library from IFN-treated mouse L929 cells under screening conditions that have been previously described (25, 35).

Cosmid clones were obtained from a library constructed as previously described (26). Vectors arms (pcos2EMBL) were ligated to *Sau*3A partially digested mouse genomic DNA, packaged in vitro, purified over a cesium chloride step gradient and plated on DH-1 cells. After overnight growth, kanamycin-resistant colonies were harvested and stably frozen at −70°C. This frozen stock was titred and used to plate approximately 2×10^5 recombinants which were screened by hybridization according to standard methods (31) using a radiolabelled 220 bp *Eco*RI-*Mst*II fragment from cDNA L3 (see Figure 1) as probe.

Northern and Southern analyses

Preparation, electrophoresis, blotting and probing of RNA and DNA samples was carried out using standard techniques (31). Northern blots were washed down to 0.1×SSC at 50°C (Figure 2) or 0.2×SSC at 42°C (Figures 5−7) and Southern blots were washed down to 0.1×SSC at 65°C. High molecular weight genomic DNA from mouse JLSV9R cells was prepared by standard methods (31). Genomic DNA from the Jackson Laboratory 129/Sv strain mouse (129/SvJ) was purchased commercially.

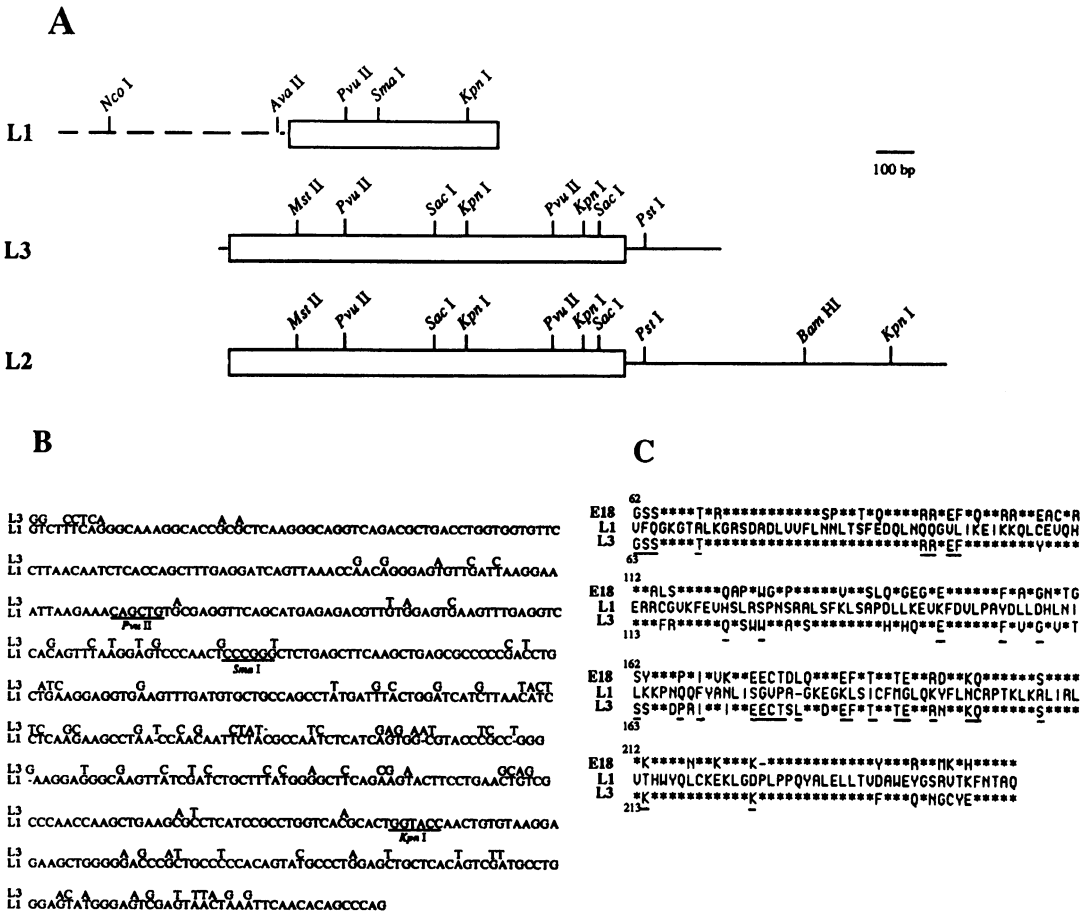

Figure 1. Novel murine 2-5A synthetase cDNAs, L1 and L2 are similar to but distinct from cDNA L3. Panel A: Restriction maps for L1, L2 and L3 are shown. Open boxes indicate open reading frames (ORFs). Solid horizontal lines represent untranslated sequences. The dashed horizontal line represents presumed intervening sequence (IVS). Regions of these cDNAs were used as probes in this study; their names and derivations are as follows. L1/IVS is a 647 bp fragment from the 5′ terminus (left end) of L1 to the AvaII site shown. L3/5′ is a 220 bp fragment from the 5′ terminus (left end) of L3 to the MstII site shown. L3/ORF is a 1.2 kb fragment from the 5′ terminus of L3 to the PstI site shown. From this PstI site to the 3′ terminus of the cDNA is a 216 bp fragment called L3/3′ut. L2/3′ut is a 400 bp fragment from the *Bam*HI site to the 3′ terminus (right end) of L2. Panel B: The ORF nucleotide sequence of L1 is shown. The sequences for L3 and L1 are the same except where differences have been indicated above the L1 sequence. The L3 sequence begins at nucleotide 222 and ends at nucleotide 800 (numbering as in reference 24). Hyphens mark gaps introduced in the sequences to facilitate comparison. Some restriction sites are indicated below the L1 sequence. Panel C: The amino acid sequence for L1 is listed. Above this sequence the similar region (amino acids 62−253, ref. 15) of E18 is also given where asterisks indicate residues in E18 identical to L1 residues. Below L1, the similar region (amino acids 63−255, ref. 24) of L3 is listed where residues in common are also indicated by asterisks. Hyphens mark gaps introduced in the sequences to facilitate comparison. Underlined amino acids indicate where L3 and E18 have the same residue which is different from L1 at that position.

Cell culture and transfections

Monkey CV-1 cells (purchased from ATCC) and mouse JLSV9 cells (a gift from Dr.R.H. Silverman) were grown as monolayers in modified minimum essential medium supplemented to 10% (v/v) with fetal calf serum (Gibco). Homogeneous preparations of recombinant Hu-α_2-IFN (Mr 19,300) were provided by P. Trotta and T. Nagabhushan, Schering Corp., NJ, at a specific activity of 2×10^8 U/mg protein. CHO recombinant human IFNβ was a gift from Interpharm Laboratories, Ness Ziona, Israel. Mouse IFNα/β was supplied by Lee Biomolecular.

Transfections were performed as previously described (32). Cosmid-transfected cells received 45 μg cosmid DNA plus 1 μg pSVtk*neo*β (33). Mock-transfected cells received no DNA in transient assays but in stable assays received 1 μg pSVtk*neo*β and 20 μg pUC18 per 10 cm dish. Pools of stably-transfected cells were developed as follows. After incubation with DNA, the cells were washed, glycerol shocked and left in medium containing 5 mM sodium butyrate for 12 h (34). Cells were then carried in fresh medium containing 400 μg/mL bioactive Geneticin (Gibco) until stable colonies were visible. These colonies were collected into pools which were analyzed as described in Figure 7.

RESULTS

Novel murine 2-5A synthetase cDNAs

cDNA clones encoding the low molecular weight form of murine 2-5A synthetase have been described by several laboratories. The first cDNA isolated, J-2 (23), is a partial cDNA. The full-length sequence was subsequently described (24) and a further identical full length cDNA isolated and referred to as L3 (25, 35). We have isolated two additional cDNAs termed L1 and L2 which are closely related to but distinct from L3 and from one another.

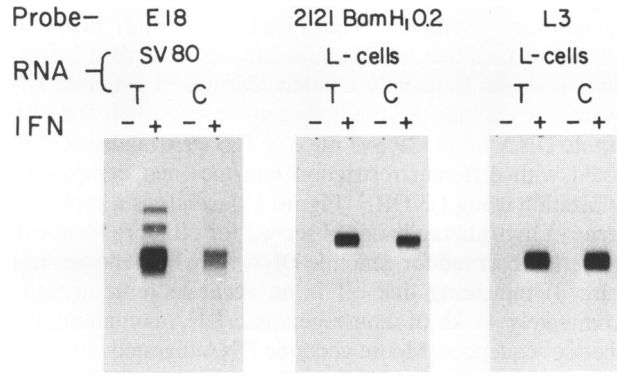

Figure 2. The L2 mRNA is IFN-inducible and slightly larger than the L3 transcript. L cells (mouse) were treated with mouse IFNα/β at 500 U/mL and SV80 cells (human) with 500 U/mL human IFNβ for 12 h. Total (T) and cytoplasmic (C) polyadenylated RNA were prepared from cells treated with media only (–) or with media containing IFN (+). A northern blot of L-cell RNA (8 μg per lane) was probed with L2/3'ut (2121*Bam*HI0.2) which detected the 2.2 kb, L2 transcript in total and cytoplasmic RNA from IFN-treated cells (middle panel). This blot was stripped and then probed with a full-length L3 probe which detected the 1.7 kb, L3 transcript in total and cytoplasmic RNA from IFN-treated cells (right panel). For comparison, a northern blot of SV80 RNA (8 μg per lane, electrophoresed and blotted along with L-cell RNA) was probed with a human 2-5A synthetase cDNA probe (E18; ref.15, 16) which, as expected, detected 1.6, 1.8, 2.7, and 3.6 kbtranscripts in total RNA from IFN-treated cells (left panel). The sizes of the various transcripts were determined using the positions of ribosomal RNAs as standards. Each blot was washed down to 0.1×SSC at 50°C. Autoradiography was for 20 h.

The cDNA L1 was isolated from a JLSV9R library screened with the human 1.8 kb cDNA (E18) as a probe. The same screening of this library produced J-2 (the incomplete version of L3) which was approximately seven times more abundant than L1 in the library(23). A shorter version of L1 was also isolated from another library prepared from S-phase mouse embryo fibroblasts (MEF) (22). The restriction map of L1 differs from the map for L3 (Figure 1, panel A) and sequence analysis (EMBL accession number, X55982) revealed that the 5', 680 bp of L1 (Figure 1, panel A) bear no similarity to any sequence of L3. This 5' region also failed to detect any transcribed sequences in northern analyses of total RNA from JLSV9R cells treated for 5–24 h with IFN (data not shown). However, as shown below, this sequence does map to cloned murine 2-5A synthetase genes and likely represents intervening sequences (IVS) transcribed to produce precursor-L1 mRNA but subsequently removed by splicing to produce a mature L1 transcript. The 3', 597 bp of this clone are 82% similar to nucleotides 222–800 of cDNA L3 (Figure 1, panel B). In northern analyses of mRNA from IFN-treated JLSV9R cells this portion of L1 recognizes a 1.7 kb transcript which appears 4 h after treatment with IFN and an approximately 4 kb transcript which appears after 18 h IFN treatment (data not shown). These transcripts are also recognized using L3 as a probe (data not shown). The open reading frame (ORF) encoded by the 3', 597 bp of L1 is 70% similar to amino acids 63–255 of the protein predicted by L3 (Figure 1, panel C; 24). At 32 of the 57 positions where the ORF of L1 is different

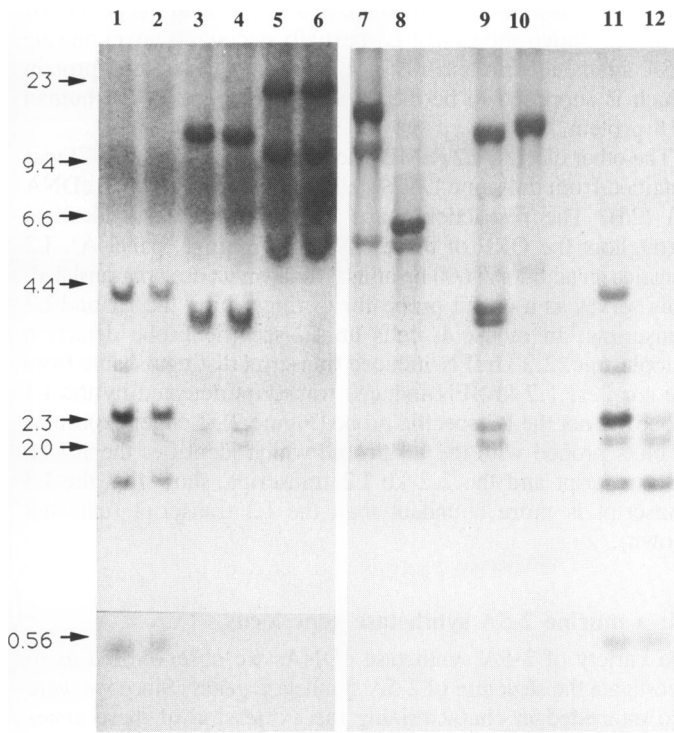

Figure 3. Cosmid clone cII is an accurate representation of murine genomic 2-5A synthetase sequences. Genomic DNAs from murine JLS-V9 cells (lanes 1,3,5), 129/SvJ mice (lanes 2,4,6), cosmid cII (lanes 7,9,11) and cosmid subclone cIIfA (lanes 8,10,12) were digested with *Bam*HI (lanes 1,2,11,12), *Hind*III (lanes 3,4,9,10) or *Eco*RI (lanes 5,6,7,8). DNA fragments hybridizing to 2-5A synthetase sequences were visualized in a Southern analysis using L3/ORF as probe. Autoradiography was for 36 h (lanes 1–6) or for 3 minute (lanes 7–12). The sizes of molecular weight markers (*Hind*III digested lamda DNA) are indicated at the left in kilobases.

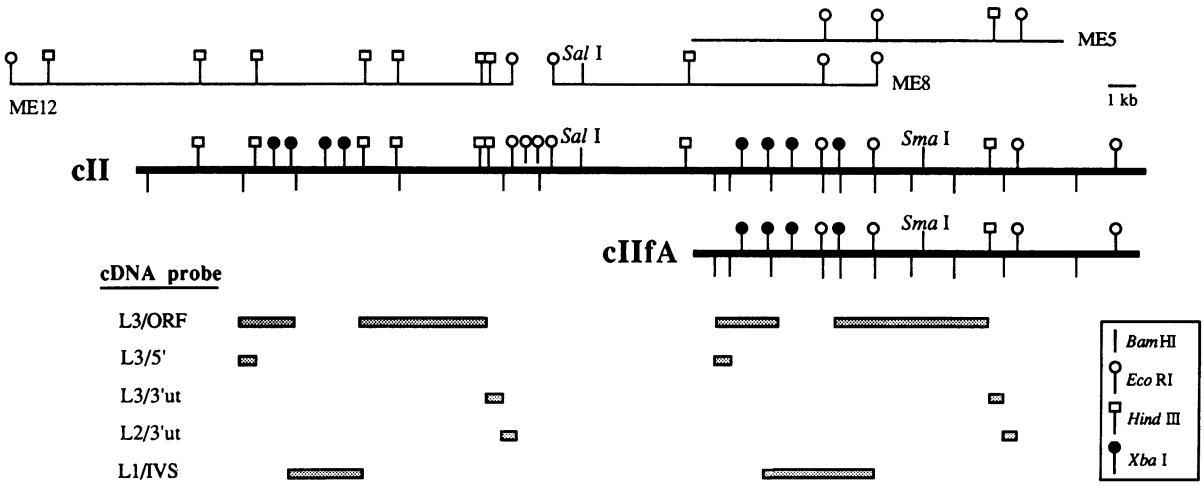

Figure 4. cII contains two murine 2-5A synthetase genes. Restriction maps for the genomic DNA contained in cII and cIIfA are shown. Symbols represening the positions of the sites for various restriction enzymes are explained in the box at the lower right . The overlaps with phage clones ME12, ME8 and ME5 (25) are shown above the cII map. The positions of two *Eco*RI sites on the approximately 8 kb central *Hind*III fragment of cII were not determined precisely therefore the symbol for *Eco*RI is not connected to the map in the region of these sites. The *Bam*HI map for cII is not complete in the ME12 region or in the region contained by the 8 kb *Hind*III fragment. Below the cosmid maps, horizontal, shaded boxes represent regions of cII and cIIfA that hybridize with the various cDNA probes indicated at the lower left side. The derivation of these probes is described in the legend to Figure 1.

from the ORF of L3, there is agreement between the ORF of L3 and the E18-predicted amino acid. The ORF in L1 continues to the 3′ end of this clone suggesting that it is incomplete at this end as well as at the 5′ end where it is joined with the presumed IVS. The above shows that L1 partially encodes a novel murine 2-5A synthetase protein distinct from the L3-encoded protein which is supposed to be the murine homologue of the human E18 protein.

The other cDNA, L2 (EMBL accession number, X58077) was obtained from the same L-929 cell library used to isolate cDNA L3 (25). The restriction maps of L2 and L3 are identical throughout the ORF of these cDNAs (Figure 1, panel A). L2 contains an additional 600 bp at its 3′ end which are not translated. This serves as a useful probe for distinguishing the L2 and L3 transcripts. In mouse L cells an L2-specific probe detects a cytoplasmic, 2.2 kb IFN-induced transcript distinguishable from the smaller, 1.7 kb IFN-induced transcript detected by the L3 probe but not the L2-specific probe (Figure 2). Longer exposures of blots probed with the L3 probe, which identifies the 1.7 kb L3 transcript and the 2.2 kb L2 transcript, show that the L3 transcript is more abundant than the L2 transcript (data not shown).

cII: a murine 2-5A synthetase gene locus

The variety of 2-5A synthetase cDNAs we observed led us to investigate the structure of 2-5A synthetase genes. Since we were also interested in characterizing the expression of these genes in cell transfection assays, the genes were isolated in cosmid vectors suitable for transfection experiments. A pcos2EMBL cosmid library containing *Sau*3A partially digested DNA from 129/Sv mice (26) was screened with a probe from the 5′ end of cDNA L3. Twenty-four positives were obtained which fell into three classes based on the observed digestion patterns. Class I (1 clone) and classIII (18 clones) positives were subsequently shown to be rearranged when compared to genomic DNA from 129/SvJ mice (data not shown); DNA from these cosmids served as negative controls in transfection experiments discussed below. Class II (5 clones) positives do not appear to be rearranged (see below) therefore a representative of this class (cII) was chosen for further analysis. We noted that digestion of cII with *Sal*I and *Mlu*I divided this cosmid into two high molecular weight bands each of which hybridized to probes derived from the 5′ and 3′ ends of cDNA L3 (Figure 1, legend) suggesting that this cosmid contained two, 2-5A synthetase genes. The larger molecular weight fragment containing most of the pcos2EMBL vector was blunt-ended, recircularized and used to transform DH5α cells to kanamycin resistance. The resulting cosmid, cIIfA was employed to facilitate the mapping studies described below.

Since cosmids frequently contain rearranged genomic DNA we wanted to exclude this possibility in the case of cII and cIIfA. Genomic DNA from 129/SvJ mice or JLSV9R mouse cells was digested with different restriction enzymes and compared by hybridization using L3/ORF (Figure 1, legend) as a probe. The patterns of hybridizing bands observed for cII were identical to the patterns observed for genomic DNA from both mouse strains (Figure 3) indicating that cII is an accurate reproduction of approximately 41 kb of mouse genomic DNA containing 2-5A synthetase sequences. Mouse genomic DNA digested with *Eco*RI contained a 23 kb band. Although this band appears to be missing in *Eco*RI-digested cII DNA, a truncated version of it containing a junction with vector sequences is present as the largest band in lane 7 (Figure 3). Mouse genomic DNA digested with *Eco*RI contained a weakly hybridizing, 1 kb band that could be visualized only after 2 days of autoradiography but was not observed for *Eco*RI digested cII. The origin of this band is not clear at present. In addition, cIIfA which was derived from cII was not rearranged during the manipulations and sub-cloning. This is evident because the hybridizing bands seen for cIIfA are subsets of the cII bands (Figure 3). The 7.6 kb *Eco*RI band (lane 8) and the 14.6 kb *Hind*III band (Lane 10) observed for cIIfA are exceptions because they contain junctions between genomic and vector DNA (data not shown). cIIfA is an accurate representation of approximately half of the 41 kb region contained in cII.

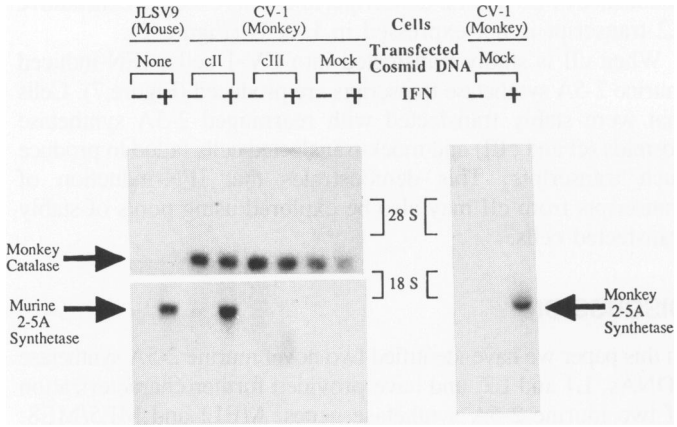

Figure 5. cII produces IFN-inducible murine 2-5A synthetase transcripts when it is transiently transfected into CV-1 cells. Monkey cells were mock-transfected or were transfected with murine 2-5A synthetase cosmids cII or cIII (a rearranged cosmid). Twelve hours post transfection, the cells were treated with media (−) or with media containing 1,000 U/mL human IFN-α_2 for 8 h. Total RNA was prepared from the cells and analyzed in northern blots (20 μg/lane) using three different probes. The left blot (8 lanes) shows that L3/3′ut (see legend to Figure 1) detected murine 2-5A synthetase transcripts in cII-transfected CV-1 cells and in control, murine JLSV9 cells but not in cIII- or mock-transfected CV-1 cells (lower half of the composite autoradiograph). Autoradiography with this probe was for 5 h. This blot was reprobed with a human catalase cDNA (30) and autoradiographed for 40 h. (upper half of composite autoradiograph). The right blot (2 lanes) was probed with the human E18 cDNA (15) to detect the endogenous monkey 2-5A synthetase gene after 5 h. autoradiography. Control mouse cells were treated with media(−) or media containing IFN for 5 h. In this experiment, the L2 and L3 transcripts were not electrophoretically distinguished.

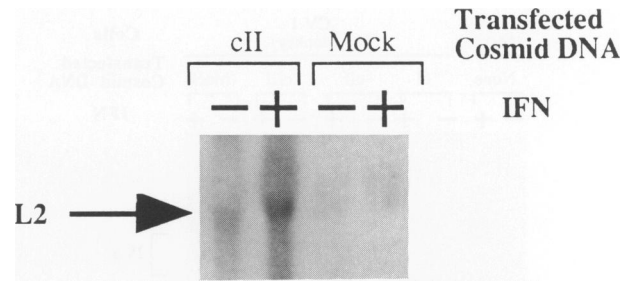

Figure 6. L2 transcripts constitute a minor portion of the murine 2-5A synthetase transcripts in cII-transfected CV-1 cells. A blot containing some of the RNA samples (20 μg/lane) from the experiment described in Figure 5 was probed with an L2-specific probe, L2/3′ut (See legend to Figure 1). cII-transfected cells displayed constitutive (−) and IFN-induced (+) L2 transcripts that were not detected in mock-transfected cells. Autoradiography was for 70 h.

To construct restriction maps of the genomic DNA contained in cII and cIIfA, linearized cII and cIIfA molecules were partially digested with *Eco*RI , *Hind*III, *Bam*HI and *Xba*I. The positions of these sites on cII and cIIfA were determined by Southern analyses of the partial digestion products using pcos2EMBL probes which hybridized to the ends of linearized, partially-digested cII and cIIfA molecules. The results of these and other mapping analyses were summarized in restriction maps of the genomic DNA contained by cII and cIIfA (Figure 4). Comparison of these maps with the restriction maps for two previously described murine 2-5A synthetase genes, ME12 and ME5/ME8 (25) showed that cII contains both of these genes (top of Figure 4). An 8 kb *Hind*III fragment in the middle of cII overlaps with the 3′ region of ME12 at one end and at its other end with the 5′ region of ME5/ME8. The 5′ region of ME12 contains an additional 4 kb not present in cII; the 3′ region of cII contains an additional 3 kb not present in ME5/ME8 (top of Figure 4). Next we determined the regions of cII and cIIfA which hybridized with cDNA L3. Three probes derived from L3 were used in Southern analyses of cII and cIIfA DNA digested with a variety of enzymes. The largest L3 probe, L3/ORF (see legend to Figure 1), broadly defined two regions, a 10 kb region for the ME12 gene and a region of similar size for the ME5/ME8 gene (bottom of Figure 4). A probe from the 5′ region of L3, L3/5′ (see legend to Figure 1), mapped to a 560 bp *Bam*HI -*Hind*III fragment in ME12 and to a 560 bp *Bam*HI fragment in ME5/ME8. A 29 bp oligonucleotide containing the interferon-responsive sequence (−80 to −67) from the ME12 gene (25) hybridized to the same fragments. A probe from the 3′, untranslated region of L3, L3/3′ut (see legend to Figure 1) defined the 3′ region of ME12 as a 500 bp *Hind*III-*Bam*HI fragment and the 3′ region of ME5/ME8 as a 600 bp *Hind*III-*Bam*HI fragment. The hybridization patterns obtained using these L3-derived probes indicate that ME12 and ME5/ME8 are likely to be complete transcriptional units each with sufficient information to produce an L3 transcript.

These transcriptional units also contain information to produce L1 and L2 transcripts. We used L1/IVS (Figure 1, legend) as an indirect guide for positioning L1/ORF on the ME12 and ME5/ME8 genes. On ME12, L1/IVS maps to a 2 kb region bordered at the 5′ end by an *Xba*I site and at the 3′ end by a *Hind*III site (bottom of Figure 4). L1/ORF therefore maps within and/or downstream of this region in ME12. On ME5/ME8, L1/IVS maps to a 4 kb region defined at the 5′ end by an *Xba*I site and at the 3′ end by an *Eco*RI site (bottom of Figure 4). Again, we expect L1/ORF lies within and/or downstream of this region. Further mapping and sequencing of ME12 and ME5/ME8 will be necessary in order to determine the exact genomic position(s) of these L1/ORF sequences with respect to the corresponding ORF present in cDNA L3.

ME12 and ME5/ME8 each contain L2-specific sequences (bottom panel, Figure 4). The probe specific for the 3′ end of L2, L2/3′ut (see legend to Figure 1), hybridizes to a 400 bp *Bam*HI -*Eco*RI fragment in ME12 and to a 600 bp *Bam*HI -*Eco*RI fragment in ME5/ME8. In each case, the fragments detected by L2/3′ut lie immediately downstream of the region detected by L3/3′ut. Given the order of these probes in cDNA L2, the order detected on the genes is not surprising.

cII contains functional 2-5A synthetase genes

Transfection assays can be used to determine the relative production of L1, L2 and L3 transcripts from ME12 or ME5/ME8 in vivo. However, before attempting these experiments it was important to show that cII could function in transfection assays. The criterion we used to test function was to transfect cII into monkey cells (CV-1) and determine whether mature, IFN-inducible 2-5A synthetase transcripts were produced. To detect these transcripts, we used murine 2-5A synthetase cDNA probes L3/3′ut and L2/3′ut as these would not cross-hybridize with any transcripts produced by the endogenous 2-5A synthetase gene(s) of the receipient monkey cells.

Monkey cells transiently transfected with cII produced an IFN-induced transcript detected by probe L3/3′ut that was indistinguishable from the transcript produced in IFN-treated murine (JLSV9) cells (Figure 5). This transcript was not detected

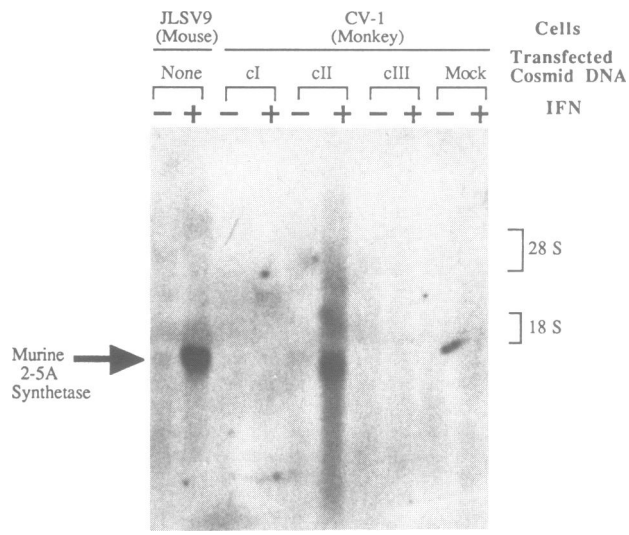

Figure 7. Pools of stable, cII-transfected CV-1 cells produce IFN-inducible murine 2-5A synthetase transcripts. CV-1 cells were mock-transfected or were transfected with cII, cI or cIII (the latter two cosmids were rearranged). In addition, each transfection coctail contained pSVtk*neoβ* which allowed for the G418-selection of cells that had taken up DNA. Pools of stably transfected (G418-resistant) CV-1 cells were treated with media (−) or with media containing 1,000 U/mL IFN-a_2 (+) for 8 h. Total RNA was prepared from cells and analyzed in a northern blot (20 μg/lane) using L3/3'ut as probe (see legend to Figure 1). Murine 2-5A synthetase transcripts were detected in cII-transfected CV-1 cells and in control, murine JLSV9 cells but not in cI-, cIII- or mock-transfected CV-1 cells. Control mouse cells were treated with media (−) or media containing IFN (+) for 5 h. Autoradiography was for 24 h.

in mock-transfected cells indicating it was not the product of any monkey genes. cIII-transfected CV-1 cells also did not produce the transcript. As mentioned above, cIII is a rearranged cosmid containing murine 2-5A synthetase sequences which hybridize to L3/3'ut and other cDNA probes derived from L3. The IFN-inducible nature of the transcript in cII-transfected cells was confirmed by analysing the levels of monkey catalase mRNA in the same cII-transfected cells. Thus the induction observed is not the result of unequal gel loading. Moreover, the transfection procedure did not affect IFN-induction of monkey 2-5A synthetase gene(s) indicating that IFN-induction of the transfected genes occured as a result of the endogenous signal transduction pathway.

The L3/3'ut probe used above detects L3, L2 (and possibly L1) transcripts. We used the L2/3'ut probe to discern the proportion of the murine 2-5A synthetase transcripts in cII-transfected cells that were L2. The L2/3'ut probe recognized an IFN-induced transcript in cII-transfected cells but not in mock-transfected cells (Figure 6). However the abundance of the L2-specific transcript was low compared to the transcripts detected using the L3/3'ut probe. There is constitutive expression of the L2 transcript in cII-transfected cells. We also noted low constitutive expression of the transcript recognized by L3/3'ut in cII-transfected cells (Figure 5), but this constitutive transcript is likely an L2 transcript recognized by the L3/3'ut probe. Taken together, these results show that the majority of IFN-induced 2-5A synthetase transcripts in cII-transfected CV-1 cells are the L3 type. The constitutive 2-5A synthetase transcripts in these cells are likely to be L2 and the level of L2 transcripts are also increased with IFN treatment. This situation reflects the relative abundance of L3 and L2 transcripts in L-cells except a constitutive L2 transcript is not expressed in L-cells (Figure 2).

When cII is stably transfected into CV-1 cells, IFN-induced murine 2-5A synthetase transcripts are produced (Figure 7). Cells that were stably transfected with rearranged 2-5A synthetase cosmids (cI and cIII) and mock-transfected cells failed to produce such transcripts. This demonstrates that IFN-induction of transcripts from cII may also be explored using pools of stably transfected cells.

DISCUSSION

In this paper we have identified two novel murine 2-5A synthetase cDNAs, L1 and L2, and have provided further characterization of two murine 2-5A synthetase genes, ME12 and ME5/ME8. These genes have similar structure because they have the same linear order of regions which hybridize with L3, L1-specific and L2-specific probes. The L3/ORF probe used in this study cross hybridizes with L1/ORF sequences and at this level of resolution it can not be determined whether L3/ORF or L1/ORF is restricted to ME12 or ME5/ME8. However, L1/ORF contains a single *Sma*I site and only one *Sma*I site is present in cII, within the ME5/ME8 gene downstream of the L1/IVS-hybridizing region (Figure 4). Thus it is likely that L1/ORF is restricted to the ME5/ME8 gene. This could explain why thus far we have not been able to obtain a full-length L1 cDNA. Reverse transcribed from the ME5/ME8 gene transcripts, full-length L1 cDNAs are likely to contain internal *Eco*RI sites (Figure 4). These sites may have been inefficiently methylated during preparation of the *Eco*RI libraries we screened. Constructing cDNA libraries from ME5/ME8 transcripts using phage vectors (27) with a *Sal*I cloning site should overcome this problem since unlike *Eco*RI, this enzyme does not cut within the ME5/ME8 gene.

It is interesting that murine cDNAs with similar but distinct sequences were isolated. This is not the case in humans where only one gene has been cloned, mapped to human chromosome 12 and shown to encode the 40 kd and 46 kd proteins which are identical except for their distinguishing hydrophobic (E16) or acidic (E18) C-termini (14, 15, 16, 28). In immunoblotting studies, antibodies raised against a peptide (B) common to these proteins also recognize proteins of 69 kd and 100 kd (19). Recently antibodies raised against the human 69 kd and 100 kd proteins have been used to screen human cDNA libraries and clones have been isolated with 65−70% similarity at the nucleotide level with exons 3−5 of the E16/E18 gene (29). These cDNAs detect large IFN-induced transcripts but also weakly recognize the E18 mRNA and are probably the products of a second human 2-5A synthetase gene (29). It is not yet clear whether the large molecular weight transcripts detected using E16 and E18 probes in RNA from IFN-treated human cells are products of this second gene or represent precursor mRNA from the E16/E18 gene. The hybridization characteristics observed for some of these large transcripts suggest the latter (14).

The situation for murine synthetases is likely to be as complex since three IFN-induced synthetase proteins (40 kd, 75 kd and 100 kd) have been described that are recognized in mouse cells by the anti-peptide B antibodies (20). Murine 40 kd almost certainly is the product of L3 because a murine mRNA approximately the same size as L3 can be translated in *Xenopus* oocytes into a 2-5A synthetase of approximately 40 kd (10). Translated in rabbit reticulocyte lysates, in vitro transcribed RNA from L3 produces a 43 kd protein (35). Murine cells do not

appear to have a 46 kd synthetase protein because no murine synthetase cDNA has been described which is related to L3 in the same way that E18 is related to E16. It is interesting to note that L3 encodes a protein with a C-terminus in which 8 out of 19 amino acids are identical with the C-terminus of the human 46 kd synthetase (24) even though the L3-encoded protein is closer in size to the human 40 kd synthetase. Perhaps the murine L3 product can assume the functions of both the 40 kd and 46 kd human proteins. This begs the question as to the origin of L1 and L2. It is possible that L1 represents a transcript which encodes a 40 kd type of protein and perhaps ME12 and ME5/ME8 are simply duplicated genes with one type of product, 40 kd synthetase proteins. Thus, other, yet to be described murine synthetase genes would encode the 69 kd and 100 kd proteins. Such genes might be the source of the weakly hybridizing 1 kb *Eco*RI band we detected in mouse genomic DNA but not in cII DNA using the L3/ORF probe. Alternatively, because the L1-predicted protein differs from the L3-predicted protein at a number of positions (32 out of 193) which are conserved between L3 and the human E18 proteins, L1 may represent a transcript which encodes one of the large synthetase proteins. Other, indirect evidence supports this view. For example, two of our L1-type cDNAs were cloned from murine JLSV9R cells that produced an approximately 4 kb IFN-induced transcript easily detectable by northern analysis of 10 μg of total RNA. In most other mouse cell lines we have examined, 5–10 μg of poly(A)$^+$ RNA is required in order to detect the large transcript by this technique. Another L1-type cDNA was cloned from a library prepared from S-phase MEF cells in which large (4–5 kb) 2-5A synthetase transcripts are more abundant than the 1.7 kb IFN-induced transcript (22). Furthermore, we noted that the 4 kb transcript in JLSV9R cells appeared only after 18 h of treatment with IFN, whereas the 1.7 kb transcript appears as early as 4 h after IFN treatment. In human, IFN-treated Daudi cells, the 100 kd synthetase protein also appears later than the 46 kd synthetase (20) thus it remains possible the 4 kb transcript might encode the 100 kd mouse synthetase. In several human cell lines, constitutive levels of the 100 kd synthetase are detectable (19, 20). The ME12 and ME5/ME8 promoters appear to have different constitutive activities both in COS-7 and NIH/3T3 cells (25). In both lines, the 600 bp ME5/ME8 promoter fragment has approximately 2 to 5 times more constitutive activity than the comparable 600 bp fragment from ME12. Thus the ME5/ME8 gene may consitutively produce the L1 transcript encoding the 100 kd synthetase.

The function served by the transcripts represented by L2 is unclear. The protein predicted by L2 is the same size as that predicted to be encoded by L3 therefore the two proteins are not easily distinguished in vivo. RNA transcribed from cDNA L2 and translated in vitro yielded synthetase protein of the expected size which lacked enzyme activity and sequence analysis of L2 revealed mismatches with L3 and L1 (J. Chebath, unpublished results). A further understanding of the relation between the ME genes and the L1, L2 and L3 transcripts will require more detailed structural analysis of the two genes and the development of further gene specific probes or use of PCR. However, the transfection studies reported in this paper show that cII-transfected CV-1 cells produce L3 and L2-specific IFN-inducible transcripts and each is IFN-inducible as is the case in mouse cells. The presence of a constitutive, L2 transcript in the transfected CV-1 cells might reflect tissue-specific expression of this transcript. The production of large murine synthetase transcripts from cII will require investigating alternative hosts since CV-1 cells do not produce easily detectable (by northern analysis) levels of large 2-5A synthetase transcripts. However other human cell lines such as HeLa, SV80 and FS11 all produce large 2-5A synthetase transcripts (Figure 2, left panel; 14,15) and may be useful for analysing the production of large murine 2-5A synthetase transcripts from cII DNA. It remains to transfect ME12 and ME5/ME8 individually in these cells to determine if either gene produces the large transcript exclusively.

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